

AD _____

Award Number: DAMD17-00-1-0559

TITLE: Phosphoinositide 3-Kinase/AKT1 Pathway and Human Ovarian Cancer

PRINCIPAL INVESTIGATOR: Jin Q. Cheng, Ph.D.

CONTRACTING ORGANIZATION: University of South Florida
Tampa, Florida 33620

REPORT DATE: September 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20021001 054

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 2001	3. REPORT TYPE AND DATES COVERED Annual (01 Sep 00 - 31 Aug 01)	
4. TITLE AND SUBTITLE Phosphoinositide 3-Kinase/AKT1 Pathway and Human Ovarian Cancer			5. FUNDING NUMBERS DAMD17-00-1-0559	
6. AUTHOR(S) Jin Q. Cheng, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of South Florida Tampa, Florida 33620 E-Mail: jcheng@hsc.usf.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Report contains color				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Extensive studies have demonstrated that the Akt pathway is essential for cell survival and anti-apoptosis; however, alterations of Akt in human malignancy have not been documented. We have recently demonstrated significantly increased AKT1 and AKT2 kinase activity in primary ovarian carcinomas. We have also shown that PI3K is frequently activated in the specimens with activation of Akt. The majority of cases with PI3K/Akt activation are late stage and high grade. The biological significance of AKT1 activation in human cancer was demonstrated by malignant transformation of NIH 3T3 cells transfected with constitutively active AKT1, but not cells transfected with wild type AKT1. Moreover, inhibition of PI3K/Akt pathway inhibits cell growth and induces apoptosis in human ovarian cancer cell lines. We have also observed that estrogen receptor (ER) α interacts and activates PI3K/Akt pathway. PI3K/Akt feedback regulates ER α by phosphorylation of serine-167 of ER α . These data indicate that AKT1 kinase, which is frequently activated in human cancer, is a determinant in oncogenesis and a potential target for cancer intervention.				
14. SUBJECT TERMS PI3K, Akt, ovarian cancer, transformation, estrogen receptor				15. NUMBER OF PAGES 43
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusions.....	5
References.....	6
Appendices.....	6

INTRODUCTION:

The purpose of this project is to: 1) Determine the incidence and clinical significance of *PI3K/AKT1* alterations in ovarian cancer; 2) Determine the role of overexpression of active and wild type PI3K and AKT1 in ovarian surface epithelial cell transformation and 3) Determine PI3K and AKT1 as targets for ovarian cancer intervention

BODY:

During the current budget year, we focused our efforts on the examination of incidence and clinical significance of PI3K/AKT1 alterations in human ovarian cancer and PI3K/AKT1 as a potential target for ovarian cancer intervention.

I. PI3K/AKT1 is frequently activated in human primary ovarian cancer.

Extensive studies have demonstrated that the Akt pathway is essential for cell survival and anti-apoptosis (1); however, alterations of Akt in human malignancy have not been documented. Total 91 ovarian tumor specimens have been examined for protein expression and kinase activity of AKT1. Activation of PI3K and AKT1 was observed in 39.3% tumor examined, however, elevated AKT1 protein level was only detected in 8.3% (2, see Appendix). We have also looked at PTEN, a tumor suppressor gene encoding dual phosphatase that dephosphorylates PI3K products (PI3,4,5P₃) and inhibits AKT1 activity (3), alterations in this series of tumors. Three cases showed PTEN down-regulation. These data suggest that PI3K activation is primary reason to result in elevated AKT1 kinase levels in human ovarian cancer. The majority of the tumors with PI3K/AKT1 activation are late stages and high grades indicating that alterations of PI3K/AKT1 are associated with tumor progression rather than initiation.

II. Expression of constitutively activated AKT1 results in malignant phenotype in NIH 3T3 cells.

It has been demonstrated that wild type AKT1 was unable to transform NIH 3T3 cells (4). To determine whether activation of AKT1 in human tumors has biological implication, we have introduced HA-tagged constitutively active forms of Akt (Myr-Akt and Akt-E40K), wild type, and myristoylated kinase-inactive mutant (Myr-Akt-K179M) Akt into NIH 3T3 cells individually. After G418 selection, 5 stable clonal cell lines from each transfection were obtained. Cells transfected with constitutively active forms of Akt, but not myr-Akt-K179M and WT-Akt were morphologically transformed, grew in medium with low serum (0.1%), formed colonies in soft agar suspension, and were highly tumorigenic in nude mice. Tumors were observed 1-3 weeks after the injection of constitutively activated Akt-transfected cells in all mice (2). These data suggest that kinase activity of Akt/AKT1 is essential for oncogenic transformation in NIH 3T3 cells.

III. Inhibition of PI3K/Akt pathway induces apoptosis in human ovarian cancer cells.

To assess the PI3K/Akt as potential target for ovarian cancer intervention, 3 ovarian cancer cell lines with or without PI 3-kinase/AKT activation were treated with PI 3-kinase inhibitors, wortmannin or LY294002, or vehicle (DMSO) for 12 hours in a medium containing 1% fetal calf

serum. Those cell lines exhibiting elevated levels of PI3K/AKT activity underwent apoptosis after treatment with wortmannin or LY294002, whereas no apoptosis was detected in the cell line without PI 3K/AKT activation (5, see Appendix).

IV. Estrogen receptor (ER) α binds to and activates PI3K/Akt pathway which feedback regulates ER α transcriptional activity.

Although the role of ER α in ovarian oncogenesis is controversial, animal experimental data showed that estrogen significantly induces ovarian surface epithelial (OSE) cell proliferation (6). We have recently demonstrated that ER α physically interacts with p85 α regulatory subunit of PI3K and induces PI3K/Akt pathway. AKT phosphorylated serine-167 of ER α *in vitro* and *in vivo* and stimulated its transcriptional activity (7, see Appendix). It has been shown that ER α can upregulate cyclin D1, c-myc, cathepsin D and TGF α that are known to stimulate cell proliferation. Therefore, regulation between PI3K/Akt pathway and ER α could contribute to control of OSE cell growth.

In addition, we collaborated with Dr. Patricia Kruk's group and identified that PI3K mediates stress-induced telomerase activity (8, see Appendix). Unfortunately, the grant number (DAMD17-00-1-0559) was forgotten putting in the paper.

KEY RESEARCH ACCOMPLISHMENTS:

1. Identification of frequent alterations of PI3K/AKT1 in human primary ovarian cancer which may associate with tumor progression.
2. Activation of AKT1 in human ovarian cancer has important biological implication in term of malignant transformation.
3. Inhibition of PI3K/AKT1 pathway induces apoptosis in human ovarian cancer cells.
4. Regulation between PI3K/Akt pathway and ER α could contribute to ovarian carcinogenesis.

REPORTABLE OUTCOMES:

1. Four manuscripts (see appendix).
2. One abstract in "17th Annual Meeting on Oncogene; Cancer Cell Signal Transduction".

CONCLUSIONS:

Our data demonstrated: 1) activation PI3K/AKT1 pathway is a common occurrence in human ovarian cancer; 2) activation of AKT1 results in malignant transformation; 3) inhibition PI3K/AKT1 pathway inhibits ovarian cancer cell growth and induces apoptosis and 4) PI3K/AKT1 regulates ER α and telomerase activity to control the OSE cell proliferation.

REFERENCES:

1. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.*, 68: 965-1014, 1999.
2. Sun M, Wang, G, Paciga, JE, Feldman RI, Yuan Z, Ma X, Shelley SA, Jove R, Tsichlis PN, Nicosia SV, Cheng J.Q. AKT1/PKB α kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am. J. Pathology* 159:431-437, 2001.
3. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P. and Mak, T. W. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 95:29-39, 1998.
4. Cheng, J. Q., Altomare, D. A., Klein, M. A., Lee, W.-C., Mysliwiec, T., Lissy, N. A., and Testa, J. R. Transforming activity and cell cycle-dependent expression of the *AKT2* oncogene: evidence for a link between cell cycle regulation and oncogenesis. *Oncogene*, 14: 2793-2801, 1997.
5. Yuan, Z., Sun, M., Feldman, R.I., Wang, G., Ma, X., Coppola, D., Nicosia, S.V. and Cheng, J.Q. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene* 19:2324-2330, 2000.
6. Bai, W., Oliveros-Saunders, B., Wang, Q., Acevedo-Duncan, M.. E., and Nicosia, S. V. Estrogen stimulation of ovarian surface epithelial cell proliferation. *In Vitro Cell Dev Biol Anim.* 36:657-666, 2000.
7. Sun M, Paciga, J.E, Feldman RI, Yuan Z, Shelley SA, Nicosia SV, Cheng J.Q. Phosphatidylinositol-3-OH kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor α (ER α) via interaction between ER α and PI3K. *Cancer Res.*, 61:5985-5991, 2001.
8. Alfonso-De Matte, M.Y, Cheng, J.Q., and Kruk, P.A. Ultraviolet irradiation- and dimethyl sulfoxide-induced telomerase activity in ovarian epithelial cell lines. *Exp Cell Res.* 267:13-27, 2001.

APPENDICES:

1. Sun M, Wang, G, Paciga, JE, Feldman RI, Yuan Z, Ma X, Shelley SA, Jove R, Tsichlis PN, Nicosia SV, Cheng J.Q. AKT1/PKB α kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am. J. Pathology* 159:431-437, 2001.
2. Sun M, Paciga, J.E, Feldman RI, Yuan Z, Shelley SA, Nicosia SV, Cheng J.Q. Phosphatidylinositol-3-OH kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor α (ER α) via interaction between ER α and PI3K. *Cancer Res.*, 61:5985-5991, 2001.
3. Alfonso-De Matte, M.Y, Cheng, J.Q., and Kruk, P.A. Ultraviolet irradiation- and dimethyl sulfoxide-induced telomerase activity in ovarian epithelial cell lines. *Exp Cell Res.* 267:13-27, 2001.
4. Yuan, Z., Sun, M., Feldman, R.I., Wang, G., Ma, X., Coppola, D., Nicosia, S.V. and Cheng, J.Q. Frequent activation of AKT2 and induction of apoptosis by inhibition of

phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene* 19:2324-2330, 2000.

Short Communication

AKT1/PKB α Kinase Is Frequently Elevated in Human Cancers and Its Constitutive Activation Is Required for Oncogenic Transformation in NIH3T3 Cells

Mei Sun,* Gen Wang,* June E. Paciga,*
Richard I. Feldman,[†] Zeng-Qiang Yuan,*
Xiao-Ling Ma,* Sue A. Shelley,* Richard Jove,*
Philip N. Tsichlis,[‡] Santo V. Nicosia,* and
Jin Q. Cheng*

From the Department of Pathology and Laboratory Medicine and the Molecular Oncology Program,* University of South Florida College of Medicine and H. Lee Moffitt Cancer Center, Tampa, Florida; the Cancer Research Department,[†] Berlex Biosciences, Richmond, California; and the Kimmel Cancer Center,[‡] Thomas Jefferson University, Philadelphia, Pennsylvania

Extensive studies have demonstrated that the Akt/AKT1 pathway is essential for cell survival and inhibition of apoptosis; however, alterations of Akt/AKT1 in human primary tumors have not been well documented. In this report, significantly increased AKT1 kinase activity was detected in primary carcinomas of prostate (16 of 30), breast (19 of 50), and ovary (11 of 28). The results were confirmed by Western blot and immunohistochemical staining analyses with phospho-Ser473 Akt antibody. The majority of AKT1-activated tumors are high grade and stage III/IV (13 of 16 prostate, 15 of 19 breast, and 8 of 11 ovarian carcinomas). Previous studies showed that wild-type AKT1 was unable to transform NIH3T3 cells. To demonstrate the biological significance of AKT1 activation in human cancer, constitutively activated AKT1 (Myr-Akt) was introduced into NIH3T3 cells. Overexpression of Myr-Akt in the stably transfected cells resulted in malignant phenotype, as determined by growth in soft agar and tumor formation in nude mice. These data indicate that AKT1 kinase, which is frequently activated in human cancer, is a determinant in oncogenesis and a potential target for cancer intervention. (*Am J Pathol* 2001, 159:431–437)

Akt, also known as protein kinase B, represents a subfamily of the serine/threonine protein kinases.^{1–5} Akt/AKT1/PKB α signaling has been extensively studied

throughout the last 6 years. It has been shown that Akt is activated by a variety of stimuli in a phosphoinositide-3-OH kinase (PI 3-kinase)-dependent manner.^{6–9} Activation of Akt by growth factors depends on the integrity of the PH domain, which binds to the PI 3-kinase product, PI(3,4,5)P₃, and phosphorylation of Thr-308 and Ser-473 by PDK1 and PDK2/ILK, respectively. In addition, growth factor-induced Akt activation is also mediated by Ras, Src, and Gab1.^{10–12} In numerous cell types, it has been shown that Akt induces survival and suppresses apoptosis induced by a variety of stimuli, including growth factor withdrawal and loss of cell adhesion. The mechanisms by which Akt promotes cell survival include phosphorylation of the pro-apoptotic proteins BAD, caspase-9, Forkhead transcription factors, and I κ B kinase α , resulting in reduced binding of BAD to Bcl-X_L, inhibition of caspase-9 protease activity, Fas ligand gene transcription, and activation of the nuclear factor- κ B cascade.^{13–17} Akt has also been shown to inhibit the Raf-MEK-ERK pathway through phosphorylation of Raf-1 in myotubes and overcome constitutively activated MAPK-induced cell-cycle arrest in MCF7 cells.^{18,19}

Although Akt/AKT1 is essential for cell survival and anti-apoptosis, alterations of Akt/AKT1 have not been consistently observed in any human malignancy. In fact, amplification of AKT1 has been reported in only a single gastric carcinoma.²⁰ In this communication, we describe frequent activation of AKT1 in human carcinomas of prostate, breast, and ovary. We also demonstrate the biological significance of AKT1 activation in human cancer by showing that constitutively activated, but not wild-type, Akt is highly oncogenic in NIH3T3 cells.

Supported by grants from the National Institutes of Health (CA77935) and the Department of Defense (OC990075). =DAMD17-00-1-0559

M.S. and G.W. contributed equally to this work.

Accepted for publication May 4, 2001.

Address reprint requests to Jin Q. Cheng, M.D., Ph.D. and Santo V. Nicosia, M.D., Department of Pathology and Laboratory Medicine, University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd., MDC Box 11, Tampa, FL 33612. E-mail: jcheng@hsc.usf.edu.

Materials and Methods

Tumor Specimens, Cell Lines, Transfection, and Transformation Assay

All primary human cancer specimens were obtained from patients who underwent surgery at the H. Lee Moffitt Cancer Center and each sample contained at least 70% tumor cells as was confirmed by histological examination. The tissues were snap-frozen and stored at -70°C . Slides from each case were reviewed for grade and stage following the criteria of the American Joint Committee on Cancer, 1988 edition. NIH3T3 cells were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Transfection was performed with LipofectAMINE PLUS (Life Technologies, Inc., Rockville, MD). Stable clonal cell lines were established after G418 selection. Soft agar suspension and tumorigenesis assays were performed as previously described.²¹

Plasmids

Hemagglutinin epitope (HA)-tagged wild-type, constitutively active (Myr-Akt and Akt-E40K), and dominant-negative (kinase-inactive mutant Myr-Akt-K179M) Akt were described previously.²²

Immunoprecipitation and Western Blotting Analysis

The frozen tissue was lysed by a tissue tearor in a lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mmol/L phenylmethylsulfonyl fluoride, 2 $\mu\text{g}/\text{ml}$ aprotinin and leupeptin, 2 mmol/L benzamide, 20 mmol/L NaF, 10 mmol/L NaPPi, 1 mmol/L sodium vanadate, and 25 mmol/L β -glycerophosphate. An equal amount of protein was analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 minutes. After removal of the beads by centrifugation, lysates were incubated with the indicated antibody in the presence of protein A-protein G (2:1) agarose beads for 2 hours at 4°C . The beads were washed once with 50 mmol/L Tris-HCl (pH 7.5), 0.5 mol/L LiCl, 0.5% Triton X-10, twice with phosphate-buffered saline (PBS), and once with 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl_2 , 10 mmol/L MnCl_2 , and 1 mmol/L dithiothreitol, all containing 20 mmol/L β -glycerophosphate and 0.1 mmol/L sodium vanadate. Immunoprecipitates were subjected to *in vitro* kinase assays or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates with phospho-Ser473 Akt (New England Biolabs, Beverly, MA) or anti-AKT1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Detection of antigen-bound antibody was performed with the ECL Western blotting analysis system (Amersham, Arlington Heights, IL).

Immunohistochemistry

Formalin-fixed paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded alcohol to distilled water. The sections were subjected to antigen retrieval by boiling in a microwave for 30 minutes in 0.01 mol/L sodium citrate buffer (pH 6.0) and then exposed to 3% hydrogen peroxide diluted in water for 20 minutes at room temperature to block endogenous peroxidase activity. Sections were incubated in a blocking solution (PBS containing blocking serum) for 20 minutes followed by 20 minutes with an avidin/biotin blocking solution (Vector Laboratories, Burlingame, CA). The primary antibody to phospho-S473 Akt (Upstate Biotechnology, Lake Placid, NY) or PTEN (Upstate Biotechnology) was applied and incubated overnight at 4°C . After incubation, the slides were treated with biotinylated secondary antibody, washed, and treated with streptavidin and biotinylated horseradish peroxidase according to the manufacturer's instruction (Vector Laboratories, Burlingame, CA). After washing, the signal was visualized by diaminobenzidine tetrahydrochloride. A negative control reaction with no primary antibody was always performed alongside the reaction-containing sample.

In Vitro Protein Kinase Assay

In vitro Akt kinase assays were performed as previously described.¹¹ Briefly, the reaction was performed in the presence of 10 μCi of [γ - ^{32}P]ATP (New England Nuclear, Boston, MA) and 3 $\mu\text{mol}/\text{L}$ of cold ATP in 30 μl of buffer containing 20 mmol/L Hepes (pH 7.4), 10 mmol/L MgCl_2 , 10 mmol/L MnCl_2 , and 1 mmol/L dithiothreitol. Histone H_2B was used as the exogenous substrate. After incubation at room temperature for 30 minutes, the reactions were stopped by adding protein-loading buffer, and the products were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

PI 3-Kinase Assay

Anti-p85 (Santa Cruz) antibody was used to immunoprecipitate p110 catalytic subunits of PI 3-kinase from the tumor lysate. The immunoprecipitates were washed once with cold PBS, twice with 0.5 mol/L LiCl, 0.1 mol/L Tris (pH 7.4), and finally with 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid. The presence of PI 3-kinase activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mmol/L HEPES (pH 7.4), 10 mmol/L MgCl_2 , 50 $\mu\text{mol}/\text{L}$ ATP, 20 μCi [γ - ^{32}P]ATP, and 10 μg L- α -phosphatidylinositol-4,5-bis phosphate (PI-4,5-P₂; Biomol, Plymouth Meeting, PA) or L- α -phosphatidylinositol-4-phosphate (Sigma Chemical Co., St. Louis, MO) for 20 minutes at 25°C . The reactions were stopped by adding 100 μl of 1 mol/L HCl. Phospholipids were

extracted with 200 μ l of $\text{CHCl}_3\text{CH}_3/\text{MeOH}$. Phosphorylated products were separated by thin-layer chromatography as previously described.²³ The conversion of PI-4,5- P_2 to PI-3,4,5- P_3 and PI-4- P_1 to PI-3,4- P_2 was determined by autoradiography and quantitated by using a PhosphorImager.

Results

Frequent AKT1 Activation in Human Cancer

Despite the fact that the PI 3-kinase/Akt/AKT1 pathway is essential for cell survival and anti-apoptosis, consistent alterations of AKT1 in human primary tumors have not been well documented. We have previously examined AKT1 and AKT2 alterations at the DNA and/or mRNA levels in more than 100 cancer cell lines (including NCI 60 cancer cell lines screen) and more than 300 primary tumors from various organs. Amplification and/or overexpression of AKT2 were observed in 15 to 25% of ovarian and pancreatic tumors examined,^{2,24,25} whereas no AKT1 alteration at the DNA or mRNA level was detected (J. Q. Cheng and J.R. Testa, unpublished data). The essential role of AKT1 kinase in cell survival prompted us to examine if AKT1 kinase activity is elevated in human cancer. We first examined whether anti-AKT1 antibody (D-17, Santa Cruz) specifically recognizes AKT1. HEK293 cells were transiently transfected with HA-AKT1,

Table 1. Frequencies of AKT1 Activation and Tumor Stage and Grade

		Kinase activity		
	<i>n</i>	Normal	High	<i>P</i> value
Stage				
Prostate cancer				
T1-T2	12	9	3	0.013 [§]
T3-T4	18	5	13	
Breast cancer				
I-II	19	15	4	0.038 [§]
III-IV	31	16	15	
Ovarian cancer				
I-II	9	7	2	0.155
III-IV	19	10	9	
Grade				
Prostate cancer				
<7*	16	10	6	0.055
>7	14	4	10	
Breast cancer				
1-2 [†]	16	14	2	0.009 [§]
3 [‡]	34	17	17	
Ovarian cancer				
1-2 [†]	11	8	3	0.186
3 [‡]	17	9	8	

*Gleason score.

[†]Well (1) or moderately (2) differentiated.

[‡]Poorly differentiated.

[§] $P < 0.05$.

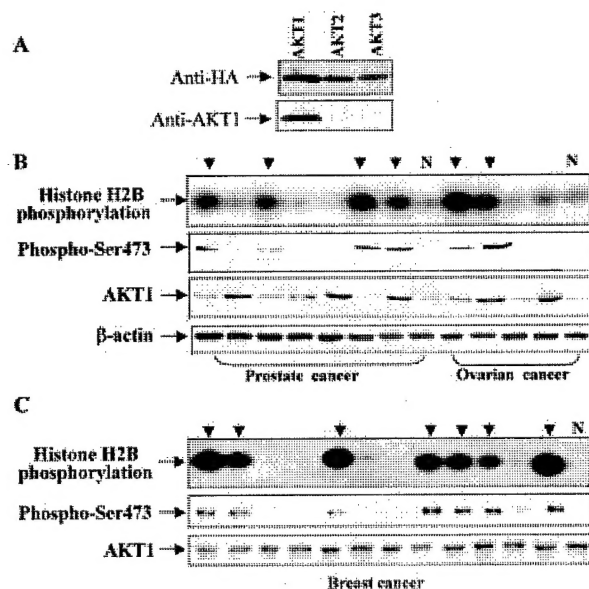


Figure 1. Activation of AKT1 in human cancers. **A:** Western blot. HEK293 cells were transiently transfected with HA-AKT1, HA-AKT2, or HA-AKT3 expression construct, lysed, immunoprecipitated with anti-HA antibody, and detected with anti-HA (top) or anti-AKT1 antibody (bottom). The top panels of **B** and **C** are *in vitro* kinase assays of AKT1 immunoprecipitates from frozen tumor specimens of prostate and ovary (**B**), and breast (**C**). AKT1 kinase levels were highly elevated in cases indicated by arrows. Normal tissue lysates (N) from prostate, ovary, and breast were used as controls. The second panels of **B** and **C** are Western blot analyses of AKT1 immunoprecipitates probed with anti-phospho-Ser473 Akt antibody. The cases exhibiting elevated AKT1 kinase activity displayed phosphorylation bands. The third and fourth panels of **B** and bottom panel of **C** are Western blot analyses with anti-AKT1 and anti- β -actin antibodies.

HA-AKT2, or HA-AKT3, lysed, and immunoprecipitated with monoclonal anti-HA antibody. The immunoprecipitates were separated and detected with AKT1 antibody. Figure 1A showed that anti-AKT1 antibody only reacted with HA-AKT1. We next immunoprecipitated AKT1 from lysates prepared from frozen tumors of prostate, breast, and ovary with anti-AKT1 antibody. The AKT1 immunoprecipitates were subjected to *in vitro* kinase assays. Significantly increased AKT1 kinase activity was observed in 16 of 30 prostate adenocarcinomas, 19 of 42 ductal breast cancers, and 11 of 23 ovarian serous adenocarcinomas. No elevated AKT1 activity was detected in eight lobular breast carcinomas, three endometrioid, and two borderline ovarian cancers examined, implying that alteration of AKT1 kinase level primarily involves ductal breast and serous ovarian carcinomas. Moreover, we observed that the majority of AKT1 activated-tumors are high grade and stage III/IV (Table 1), suggesting that activation of AKT1 plays an important role in tumor progression rather than initiation.

It has been shown that phosphorylation of threonine-308 and serine-473 is required for activation of AKT1 and that phospho-Ser473 Akt antibody recognizes only the phosphorylated/active form of Akt.⁹ To confirm the results obtained from *in vitro* kinase assays, we performed Western blotting analysis with this antibody. To avoid the possibility of cross-reaction of the phospho-Ser473 antibody with other isoforms of Akt/PKB family, the tissue lysates were first immunoprecipitated with the specific anti-AKT1 antibody. The AKT1 immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with anti-phospho-Ser473 antibody. Phosphorylated AKT1 was detected only in the

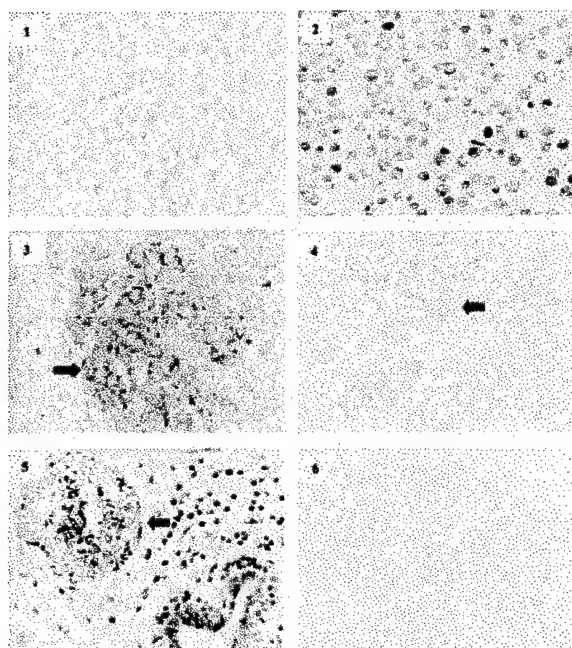


Figure 2. Phospho-AKT1 was detected in tumor cells and located only in cytoplasm. Immunohistochemical staining of the paraffin sections prepared from serum-starved (1), EGF-stimulated MCF7 cells (2), and adenocarcinomas of ovary (3), prostate (4), and breast (5 and 6) with phospho-S473 Akt antibody. Strong staining was observed in EGF-stimulated MCF7 cells and ovarian, breast, and prostate tumor cells, indicated by **black arrows**. No immunoreaction was detected in unstimulated MCF7 cells (1) normal prostate gland and ductal epithelial cells (4 and 5, **white arrow**), and the breast tumor without elevated AKT1 activity (6).

tumors with elevated AKT1 kinase activity (second panels of Figure 1, B and C).

To further demonstrate AKT1 activation and determine whether activated AKT1 is derived from tumor cells or stromal tissues in the tumor specimens with elevated AKT1 activity identified by *in vitro* kinase assay, immunohistochemical staining of tumor paraffin sections was performed with phospho-Ser473 Akt antibody. We first demonstrated that the phospho-Ser473 Akt antibody is capable of recognizing phosphorylated AKT1 by immunostaining paraffin sections prepared from serum-starved and serum-starved/EGF-stimulated MCF7 cells (Figure 2, panels 1 and 2). Phosphorylation status of AKT1 in these cells was confirmed by Western blot analysis with phospho-S473 Akt antibody (data not shown). The tumor paraffin sections from the 16 prostate, 19 breast, and 11 ovarian tumor specimens with elevated AKT1 kinase activity strongly immunoreacted with phospho-Ser473 Akt antibody (Figure 2, panels 3 to 5), whereas no immunostaining was observed in normal tissues and the tumor samples without increased AKT1 activity (Figure 2, panel 6). Interestingly, phosphorylated AKT1 was located in the tumor cell membrane and cytoplasm but not the nucleus, which is in conflict with the previously reported observation that activated Akt could translocate to the nucleus in ectopically Akt-overexpressing cells.^{26,27} Our data also showed that overexpression of GFP-tagged AKT1 translocates to the nucleus in NIH3T3 cells after IGF-1 stimulation (data not shown),

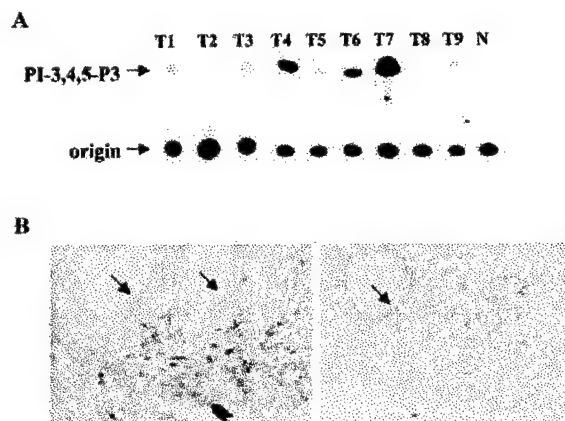


Figure 3. Activation of PI 3-kinase or down-regulation of PTEN in human tumors. **A:** *In vitro* PI 3-kinase assay of the anti-p85 immunoprecipitates from nine tumor specimens that exhibit elevated AKT1 kinase activity. Elevated levels of PI 3-kinase activity were detected in cases 4, 6, and 7. **B:** Immunostaining of paraffin sections of prostate adenocarcinomas with anti-PTEN (left) and anti-phospho-Ser473 Akt (right) antibodies. PTEN is negative in tumor cells (**black arrows**), but positive in hyperplastic glands (**arrow-head**). However, phosphorylation of Akt was only detected in tumor cells (**black arrow**).

indicating that activated Akt in the primary tumor cells could have a different subcellular localization from the cells overexpressing exogenous Akt.

Multiple Mechanisms Resulted in AKT1 Activation

Because AKT1 kinase activity is regulated positively by PI 3-kinase and negatively by *PTEN/MMAC1*,²⁸ we examined PI 3-kinase activity and PTEN expression in the tumors exhibiting AKT1 activation (Figure 3). Elevated PI 3-kinase activity was observed in 7 of the 19 breast and 5 of the 11 ovarian carcinomas, but in none of the prostate tumors that exhibit AKT1 activation. Immunohistochemical staining revealed no PTEN expression in 10 of 16 prostate and 2 of 11 ovarian cancer specimens with elevated AKT1 activity, whereas all breast carcinomas that showed AKT1 activation expressed PTEN. Absence of PTEN is well correlated with positive staining of phosphorylated AKT1 on the tumor tissue sections (Figure 3 and data not shown). In addition, we performed single-strand conformational polymorphism/sequencing analyses in AKT1-activated tumor specimens that have neither PI 3-kinase nor PTEN alteration. No AKT1 mutation was detected, implying that there are other mechanisms leading to AKT1 activation in the specimens without alterations of either PI 3-kinase or PTEN, which needs further investigation.

Constitutively Active Forms of Akt/AKT1 Are Highly Oncogenic in NIH3T3 Cells

We and others previously demonstrated that overexpression of wild-type of Akt (WT-Akt) is unable to transform NIH3T3 cells.^{21,29} To determine whether activation of AKT1 in human tumors has biological implication, we

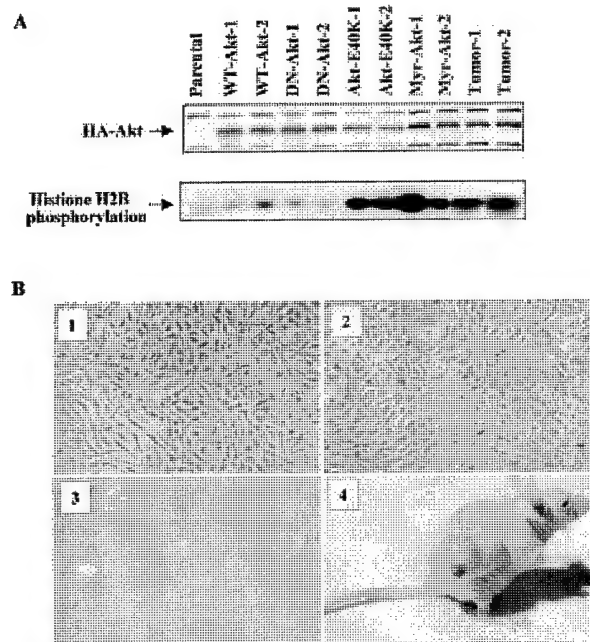


Figure 4. Constitutively activated AKT1 transforms NIH3T3 cells. **A:** Western blot (top) and *in vitro* kinase assay (bottom) analyses of Akt expression and kinase activity in stably transfected clones and a tumor sample from nude mice. For Western blot analysis, the lysates were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to membrane, and detected with anti-HA antibody. For assay of kinase activity, immunoprecipitation was performed with anti-HA antibody and the HA-Akt immunoprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate. **B:** The morphology of constitutively active Akt-transfected NIH3T3 cells (2) is more rounded and larger than WT-Akt-transfected cells (1). Constitutively activated Akt-transfected cells grew on soft agar (3) and formed tumors in nude mice (4).

have introduced HA-tagged constitutively active forms of Akt (Myr-Akt and Akt-E40K), wild-type, and myristoylated kinase-inactive mutant (Myr-Akt-K179M) Akt into NIH3T3 cells individually. After G418 selection, five stable clonal cell lines from each transfection were obtained. Western blot and *in vitro* kinase analyses revealed that all of the clonal cell lines express Akt protein (Figure 4A). High levels of kinase activity were detected in constitutively active Akt (Myr-Akt and Akt-E40K)-transfected clonal cell lines. Figure 4A shows Akt expression and kinase activity in two clonal cell lines from each transfection. Cells transfected with constitutively active forms of Akt, but not Myr-Akt-K179M and WT-Akt were morphologically transformed, grew in medium with low serum (0.1%), formed colonies in soft agar suspension, and were highly tumorigenic in nude mice (Figure 4B). Tumors were observed 1 to 3 weeks after the injection of constitutively activated Akt-transfected cells in all mice, except clone 2 of Akt-E40K cells (Figure 4 and Table 2). Although vector alone and WT-Akt-transfected NIH3T3 cells developed tumors in 1 of 25 and 2 of 25 mice, respectively, all of these tumors were detected after 38 days (Table 2). In addition, high levels of Myr-AKT1 protein and kinase activity were observed in dissected tumors from Myr-Akt nude mice but not in the tumors from vector or WT-Akt mice (Figure 4A). These data suggest that kinase activity of Akt/AKT1 is essential for oncogenic transformation in NIH3T3 cells.

Table 2. Tumorigenicity of Akt and Control Transfectant NIH3T3 Cell Lines

Clonal cell lines	Soft agar growth*	Tumorigenicity in nude mice†	Latency (days)
Akt-E40K 1	+++	5/5	14–18
Akt-E40K 2	++	4/5	17–22
Akt-E40K 3	+++	5/5	15–21
Akt-E40K 4	+++	5/5	13–18
Akt-E40K 5	+++	5/5	13–18
Myr-Akt 1	+++	5/5	11–14
Myr-Akt 2	+++	5/5	8–10
Myr-Akt 3	+++	5/5	10–11
Myr-Akt 4	+++	5/5	7–10
Myr-Akt 5	+++	5/5	8–11
Myr-Akt-K179M (1–5)	–	0/25	
Vector control (1–5)	–	1/25	40
WT-Akt (1–5)	–	2/25	38–42

*Number of colony/60 mm plate: 1–10, +; 11–30, ++; >30, +++.
†The number of injected cells: 1×10^6 /mouse.

Discussion

Activation of oncogenic signaling proteins, such as Stat3, mitogen-activated protein kinase, and p110 α , has been demonstrated in a number of different tumors.^{30–32} Constitutively activated Stat3 and PI 3-kinase are able to induce malignant transformation.^{33,34} Recent studies showed that among the most critical tumor-cell survival pathways are those mediated by the Akt/AKT1 kinase.^{6–9} In this report, we demonstrate frequently elevated AKT1 kinase activity and phosphorylation of AKT1 in human carcinomas of breast, prostate, and ovary. Moreover, we have also shown that constitutively activated, but not wild-type, AKT1 is highly tumorigenic in NIH3T3 cells. Furthermore, the majority of AKT1-activated tumors are high grade and stage III/IV. These results indicate that activation of AKT1 is a common occurrence in human cancer, especially in more advanced tumors.

We previously demonstrated that overexpression of wild-type AKT2, but not Akt/AKT1, in NIH3T3 cells resulted in malignant transformation.²¹ Ahmed and colleagues²⁹ also showed that Akt is not tumorigenic when overexpressed in the nontumorigenic rat T-cell lymphoma cell line 5675. In contrast, *v-akt*-expressing 5675 cells were highly tumorigenic. Because *v-akt* arose by an in-frame fusion of the viral Gag and Akt, the oncogenic difference between *v-akt* and wild-type Akt/AKT1 may be because of myristoylation of the amino-terminus of *v-akt*.^{29,35} Several lines of evidence show that attachment of a membrane-targeting sequence (myristoylation/palmitoylation) to the amino-terminus of AKT1/Akt is sufficient to induce its maximal activation and that the PH domain of Akt is required for its membrane translocation and activation.⁹ We demonstrated, in this study, that overexpression of constitutively active forms of Akt (Myr-Akt and Akt-E40K) in NIH3T3 cell leads to oncogenic transformation, which supports the results obtained from chicken embryo fibroblasts and Rat1 cells.^{36,37} Taken collectively, these data suggest that the kinase activity of Akt contributes to the control of cell malignant transformation and that elevated AKT1 kinase activity plays an

important role in development and/or progression of a subset of human cancers.

Previous studies have demonstrated that all of the tumor-associated *PTEN* mutants that have been biochemically characterized result in activation of AKT1.²⁸ Recently, elevated PI 3-kinase activity has been observed in human ovarian cancer.^{32,38} As discussed above, Ras, Src, and Gab1 mediate growth factor signals to activate the PI 3-kinase/Akt pathway.¹⁰⁻¹² Therefore, activation of AKT1 in human cancer could result from Ras mutation, overexpression/active mutation of growth factor receptor(s), AKT1 mutation, and Src activation. In the present report, we showed that the majority of cases with AKT1 activation had either *PTEN* down-regulation or PI 3-kinase activation, dependent on the tumor type. Activation of PI 3-kinase was frequently detected in breast and ovarian, but not prostate, carcinomas, whereas the absence of *PTEN* protein was observed in some prostate and ovarian carcinomas. However, missense mutations of *PTEN*, although uncommon, cannot be ruled out by immunohistochemistry, because they result in formation of full-length *PTEN* proteins that may be immunostained by anti-*PTEN* antibody. Nevertheless, no AKT1 mutation was found in the tumors examined, indicating that elevated AKT1 activity in human cancer results from alterations of upstream regulators of AKT1.

Amplification and/or overexpression of AKT2, but not AKT1, have been detected in a subset of human ovarian, pancreatic, and breast cancers,^{2,24,25} suggesting that AKT2 may play a more important role in human malignancy. In the present study, frequent activation of AKT1 kinase in human cancers and malignant transformation resulting from expression of constitutively activated AKT1 provide the first evidence that AKT1 could have a role similar to that of AKT2 in human cancer. Comparison of the AKT and AKT2 protein and/or kinase levels in the same tumor will provide valuable information to better understanding of the importance of AKT1 and AKT2 in human malignancy. Expression of AKT1 and AKT2 protein and alterations of AKT2 at the kinase level in these series of specimens are currently under investigation.

Subcellular localization of activated AKT1 is controversial.⁹ Early studies on the subcellular localization of Akt/AKT1 revealed that, whereas c-akt is primarily cytosolic, v-akt is distributed equally between the cell membrane, the cytoplasm, and the nucleus in NIH3T3 cells.²⁹ Recent studies showed that nuclear translocation of AKT1 and AKT2 in HEK293 and HeLa cells follows in short succession the insulin-induced translocation of AKT1 and AKT2 proteins to the cell membrane.^{26,27} However, all of these studies were performed in AKT1- or AKT2-transfected cells. In this report, we demonstrated that activated AKT1 in human primary tumors is distributed only in the plasma membrane and the cytosol, suggesting that activated endogenous AKT1 may not translocate to nucleus. In addition, we noted that immunoreaction to the phospho-Ser-473 antibody in tumor specimens is less strong, which could be because of either weak epitope of single phosphopeptide or epitope masking in paraffin section.

In summary, the data presented in this report showed that AKT1 kinase is frequently activated in human pros-

tate, breast, and ovarian carcinomas. Elevated AKT1 kinase is an essential requirement for its oncogenic activity. These results provide the basis for understanding how the Akt pathway contributes to human oncogenesis. Further studies are required to determine the clinicopathological significance of AKT1 activation and to examine if overexpression of constitutively activated AKT1 develops prostate, breast, and ovarian tumors in transgenic mouse models using tissue-specific promoters.

Acknowledgments

We thank the Tissue Procurement, DNA Sequence, and Pathology Core Facilities at H. Lee Moffitt Cancer Center for providing cancer specimens, sequencing, and immunohistochemical staining; Chen Jiang and Kun Jiang for technical support; and Wen-Ching Lee for critical reading and comments.

References

1. Bellacosa A, Testa JR, Staal SP, Tsichlis PN: A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* 1991, 254:274-277
2. Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, Testa JR: AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci USA* 1992, 89:9267-9271
3. Jones PF, Jakubowicz T, Pitossi FJ, Maurer F, Hemmings BA: Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc Natl Acad Sci USA* 1991, 88:4171-4175
4. Jones PF, Jakubowicz T, Hemmings BA: Molecular cloning of a second form of rac protein kinase. *Cell Regul* 1991, 2:1001-1009
5. Nakatani K, Sakaue H, Thompson DA, Weigel RJ, Roth RA: Identification of a human AKT3 (protein kinase B γ) which contains the regulatory serine phosphorylation site. *Biochem Biophys Res Comm* 1999, 257:906-910
6. Franke TF, Yang SL, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN: The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 1995, 81:727-736
7. Burgering BMT, Coffey PJ: Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 1995, 376:599-602
8. Meier R, Alessi DR, Cron P, Andjelkovic M, Hemmings BA: Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase B β . *J Biol Chem* 1997, 272:30491-30497
9. Chan TO, Rittenhouse SE, Tsichlis PN: AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* 1999, 68:965-1014
10. Datta K, Bellacosa A, Chan TO, Tsichlis PN: Akt is a direct target of the phosphatidylinositol 3-kinase. Activation by growth factors, v-src and v-Ha-ras, in Sf9 and mammalian cells. *J Biol Chem* 1996, 271:30835-30839
11. Liu A-X, Testa JR, Hamilton TC, Jove R, Nicosia SV, Cheng JQ: AKT2, a member of the protein kinase B family, is activated by growth factors, v-Ha-ras, and v-src through phosphatidylinositol 3-kinase in human ovarian epithelial cancer cells. *Cancer Res* 1998, 58:2973-2977
12. Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R, Wong AJ: Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. *Proc Natl Acad Sci USA* 1997, 94:12419-12424
13. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME: Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997, 91:231-241

14. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G: Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 1997, 278:687-689
15. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC: Regulation of cell death protease caspase-9 by phosphorylation. *Science* 1998, 282:1318-1321
16. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB: NF κ B activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 1999, 401:82-85
17. Romashkova JA, Makarov SS: NF κ B is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 1999, 401:86-90
18. Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD, Glass DJ: Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* 1999, 286:1738-1741
19. Zimmermann S, Moelling K: Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* 1999, 286:1741-1744
20. Staal SP: Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. *Proc Natl Acad Sci USA* 1987, 84:5034-5037
21. Cheng JQ, Altomare DA, Klein MA, Lee W.-C., Mysliwiec T, Lissy NA, Testa JR: Transforming activity and cell cycle-dependent expression of the AKT2 oncogene: evidence for a link between cell cycle regulation and oncogenesis. *Oncogene* 1997, 14:2793-2801
22. Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, McCormick F, Feng J, Tsichlis P: Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene* 1998, 17:313-325
23. Jiang K, Coppola D, Crespo NC, Nicosia SV, Hamilton AD, Sebt SM, Cheng JQ: The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. *Mol Cell Biol* 2000, 20:139-148
24. Cheng JQ, Ruggeri B, Klein WM, Sonoda G, Altomare DA, Watson DK, Testa JR: Amplification of AKT2 in human pancreatic cancer cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. *Proc Natl Acad Sci USA* 1996, 93:3636-3641
25. Bellacosa A, DeFeo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V, Ferrandina G, Benedetti-Panici P, Mancuso S, Neri G, Testa JR: Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int J Cancer* 1995, 64:280-285
26. Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM, Hemmings BA: Role of translocation in the activation and function of protein kinase B. *J Biol Chem* 1997, 272:31515-31524
27. Meier R, Alessi DR, Cron P, Andjelkovic M, Hemmings BA: Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase B β . *J Biol Chem* 1997, 272:30491-30497
28. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW: Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998, 95:29-39
29. Ahmed NN, Franke TF, Bellacosa A, Datta K, Gonzalez-Portal ME, Taguchi T, Testa JR, Tsichlis PN: The proteins encoded by c-akt and v-akt differ in post-translational modification, subcellular localization and oncogenic potential. *Oncogene* 1993, 8:1957-1963
30. Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, Dalton WS, Jove R: Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* 1999, 10:105-115
31. Gioeli D, Mandell JW, Petroni GR, Frierson Jr HF, Weber MJ: Activation of mitogen-activated protein kinase associated with prostate cancer progression. *Cancer Res* 1999, 59:279-284
32. Shayesteh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, Collins C, Pinkel D, Powell B, Mills GB, Gray JW: PIK3CA is implicated as an oncogene in ovarian cancer. *Nat Genet* 1999, 21:99-102
33. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell R, Albanese C, Darnell Jr JE: Stat3 as an oncogene. *Cell* 1999, 98:295-303
34. Chang HW, Aoki M, Fruman D, Auger KR, Bellacosa A, Tsichlis PN, Cantley LC, Roberts TM, Vogt PK: Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. *Science* 1997, 276:1848-1850
35. Bellacosa A, Franke TF, Gonzalez-Portal ME, Datta K, Taguchi T, Gardner J, Cheng JQ, Testa JR, Tsichlis PN: Structure, expression and chromosomal mapping of c-akt: relationship to v-akt and its implications. *Oncogene* 1993, 8:745-754
36. Aoki M, Batista O, Bellacosa A, Tsichlis P, Vogt PK: The akt kinase: molecular determinants of oncogenicity. *Proc Natl Acad Sci USA* 1998, 95:14950-14955
37. Mirza AM, Kohn AD, Roth RA, McMahon M: Oncogenic transformation of cells by a conditionally active form of the protein kinase Akt/PKB. *Cell Growth Differ* 2000, 11:279-292
38. Yuan Z, Sun M, Feldman RI, Wang G, Ma X, Coppola D, Nicosia SV, Cheng JQ: Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene* 2000, 19:2324-2330

Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, Activated in Breast Cancer, Regulates and Is Induced by Estrogen Receptor α (ER α) via Interaction between ER α and PI3K¹

Mei Sun, June E. Paciga, Richard I. Feldman, Zeng-qiang Yuan, Domenico Coppola, You Yong Lu, Sue A. Shelley, Santo V. Nicosia, and Jin Q. Cheng²

Department of Pathology and Programs of Molecular Oncology and Drug Discovery, University of South Florida College of Medicine and H. Lee Moffitt Cancer Center, Tampa, Florida 33612 [M. S., J. E. P., Z.-q. Y., D. C., S. A. S., S. V. N., J. Q. C.]; Beijing Institute for Cancer Research, Beijing 100034, China [Y. Y. L.]; and Cancer Research Department, Berlex Biosciences, Richmond, California 94804 [R. I. F.]

Abstract

We have shown previously that the AKT2 pathway is essential for cell survival and important in malignant transformation. In this study, we demonstrate elevated kinase levels of AKT2 and phosphatidylinositol-3-OH kinase (PI3K) in 32 of 80 primary breast carcinomas. The majority of the cases with the activation are estrogen receptor α (ER α) positive, which prompted us to examine whether AKT2 regulates ER α activity. We found that constitutively activated AKT2 or AKT2 activated by epidermal growth factor or insulin-like growth factor-1 promotes the transcriptional activity of ER α . This effect occurred in the absence or presence of estrogen. Activated AKT2 phosphorylates ER α *in vitro* and *in vivo*, but it does not phosphorylate a mutant ER α in which ser-167 was replaced by Ala. The PI3K inhibitor, wortmannin, abolishes both the phosphorylation and transcriptional activity of ER α induced by AKT2. However, AKT2-induced ER α activity was not inhibited by tamoxifen but was completely abolished by ICI 164,384, implicating that AKT2-activated ER α contributes to tamoxifen resistance. Moreover, we found that ER α binds to the p85 α regulatory subunit of PI3K in the absence or presence of estradiol in epithelial cells and subsequently activates PI3K/AKT2, suggesting ER α regulation of PI3K/AKT2 through a nontranscriptional and ligand-independent mechanism. These data indicate that regulation between the ER α and PI3K/AKT2 pathway (ER α -PI3K/AKT2-ER α) may play an important role in pathogenesis of human breast cancer and could contribute to ligand-independent breast cancer cell growth.

Introduction

Breast cancer development and tumor growth are strongly associated with estrogens. The binding of an estrogen molecule to the ER α ³ induces a cascade of events, including the release of accessory proteins (e.g., the heat-shock proteins), increased nuclear retention, DNA binding, and the transcription of estrogen-responsive genes, such as cyclin D1, c-myc, cathepsin D, and transforming growth factor- α that are known to stimulate mammary cell proliferation (1). ER α is a member of a superfamily of nuclear receptors that act as transcription factors when bound to specific lipophilic hormones. In common with other steroid hormone receptors, the ER α has a NH₂-terminal domain with a hormone-independent transcriptional activation function (AF-

1), a central DNA-binding domain, and a COOH-terminal ligand-binding domain with a hormone-dependent transcriptional activation function (AF-2; Refs. 2, 3). In addition to its ligand, estradiol, the ER α is also activated by several nonsteroidal growth factors including EGF and IGF1 through their cell membrane receptors and cytoplasmic signaling pathways such as MAPK signal transduction pathway (3, 4). Because of the role of ER α in promoting the growth and progression of breast cancers, considerable efforts are devoted to development of reagents to functionally inactivate ER α , so as to inhibit ER α -mediated gene expression and cell proliferation. Antiestrogens such as tamoxifen and ICI 164,384 antagonize the effects of estrogens by competing with estrogen for binding to ER α . Tamoxifen or its derivative 4-hydroxytamoxifen inhibits transcriptional activation by AF-2 but not AF-1 (5). ICI 164,384, on other hand, is a complete antagonist that blocks transcriptional activation by both AF-1 and AF-2 (6). However, approximately one-third of ER α -positive breast cancers fail to respond to antiestrogen treatment, which is thought to result from growth factor-induced ER α activity through activation of protein kinases resulting in phosphorylation of ER α (7).

It has been well documented that phosphorylation of ER α is essential for the activation of ER α after stimulation with its ligand and nonsteroidal growth factors (EGF and IGF1). The phosphorylation of ER α is observed at both serine and tyrosine residues. The serine residues are the predominant modified amino acids present in ER α , and four of these (Ser-104, Ser-106, Ser-118, and Ser-167) are clustered in the NH₂ terminus within the AF-1 region. Phosphorylation of ER α at Ser-118 is mediated by the Ras/MAPK pathway; therefore, activation of the MAPK pathway enables ligand-independent transactivation of ER α (4). There is evidence showing that Ser-167 is phosphorylated by several protein kinases, including casein kinase II and pp90^{rsk}¹, which is important for DNA binding and transcriptional activation (8, 9). Phosphorylation of ER α on tyrosine 537, which is required for ER α dimerization and transactivation, by Src family tyrosine kinases *in vitro* has also been demonstrated. Moreover, protein kinase A has been shown to phosphorylate ER α at Ser-236 and regulate its dimerization (10).

In addition, recent studies (11) demonstrated that plasma membrane ER α plays a crucial role in transducing cellular signals. It has been convincingly shown that ER α activates G-protein-coupled receptor leading to the modulation of downstream pathways that have discrete cellular actions including membrane K⁺ and Ca²⁺ channel activation and induction of protein kinase C and protein kinase A kinase activity (11). A recent study (12) demonstrated that estrogen activates p38 MAPK, resulting in the activation MAPK-protein kinase-2 and subsequent phosphorylation of heat shock protein 27. ER α has been also shown to interact with IGF1R and induce IGF1R and extracellular signal-regulated kinase activation (13).

Received 5/7/01; accepted 7/15/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. DAMD17-00-1-05596

¹ Supported by Grants from NIH CA77935 and Department of Defense OC990075.

² To whom requests for reprints should be addressed, at Department of Pathology and Programs of Molecular Oncology and Drug Discovery, University of South Florida College of Medicine, H. Lee Moffitt Cancer Center and Research Institute, 12901 Bruce B. Downs Boulevard, MDC Box 11, Tampa, FL 33612. Phone: (813) 974-8595; Fax: (813) 974-5536; E-mail: jcheng@hsc.usf.edu.

³ The abbreviations used are: ER α , estrogen receptor α ; PI3K, phosphatidylinositol-3-OH kinase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; EGF, epidermal growth factor; IGF1, insulin-like growth factor-1; GST, glutathione S-transferase; HEK, human embryonic kidney.

Akt, also called protein kinase B, has been identified as a direct target of PI3K (14). All of the three members, Akt/AKT1/PKB α , AKT2/PKB β , and AKT3/PKB γ , of this family are activated by growth factors in a PI3K-dependent manner (14–16). Numerous studies (17) showed that the Akt pathway is critical for cell survival by phosphorylation of a number of downstream proteins including BAD, caspase-9, Forkhead transcription factors, IKK α , Raf, and p21-activated protein kinase. Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies such as ovarian and pancreatic cancers (18–20). In this study, we demonstrate frequent activation of AKT2 and PI3K in human breast cancer. AKT2 phosphorylates ER α at Ser-167 and activates ER α -mediated transcription in a PI3K-dependent manner. ER α binds to the p85 α subunit of PI3K in epithelial cells and activates the PI3K/AKT2 pathway in an estrogen-independent manner.

Materials and Methods

Tumor Specimens, Cell Lines, and Transfection. All of the 80 primary human breast cancer specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center, and each sample contained at least 70% tumor cells, as was confirmed by microscopic examination. The tissues were snap-frozen and stored at -70°C . ER α -negative epithelial HEK293 and COS7 cells and ER α -positive MCF7 and BG-1 cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS. The cells were seeded in 60-mm Petri dishes at a density of 8×10^5 cells/dish. Transfections were performed by calcium phosphate DNA precipitation or Lipofectamine Plus (Life Technologies, Inc.).

Immunoprecipitation and Western Blotting Analysis. The cells and frozen tumor tissues were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (volume for volume) glycerol, 1% NP40, 2 mM phenylmethylsulfonyl fluoride, 2 $\mu\text{g}/\text{ml}$ aprotinin and leupeptin, 2 mM benzamide, 20 mM NaF, 10 mM NaPP $_i$, 1 mM sodium vanadate, and 25 mM β -glycerol phosphate. Lysates were centrifuged at $12,000 \times g$ for 15 min at 4°C before immunoprecipitation or Western blotting. The protein concentration in each tissue lysate was measured, and an equal amount of protein was analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 min. After removal of the beads by centrifugation, lysates were incubated with anti-AKT2 (Upstate Biotechnology) antibody in the presence of 30 μl of protein A-protein G (2:1) agarose beads for 2 h at 4°C . The beads were washed once with 50 mM Tris-HCl (pH 7.5)-0.5 M LiCl-0.5% Triton X-10, twice with PBS, and once with 10 mM Tris-HCl (pH 7.5)-10 mM MgCl $_2$ -10 mM MnCl $_2$ -1 mM DTT, all containing 20 mM β -glycerol phosphate and 0.1 mM sodium vanadate. Immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein phosphorylation and expression were determined by probing Western blots of immunoprecipitates with anti-phospho-Akt-Ser473 (Cell Signaling) or anti-AKT2 antibody. Detection of antigen-bound antibody was carried out with the enhanced chemiluminescence Western Blotting Analysis System (Amersham).

In Vitro Protein Kinase Assay. Akt kinase assay was performed as described previously (15). Briefly, the reaction was carried out in the presence of 10 μCi of [γ - ^{32}P]ATP (NEN) and 3 μM cold ATP in 30 μl of buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl $_2$, 10 mM MnCl $_2$, and 1 mM DTT using histone H2B as substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein-loading buffer, and the mixture was separated in SDS-PAGE gels. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Phosphorimager (Molecular Dynamics).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven after dewaxing and rehydration. The Vectastain ABC Kit for sheep IgG (Vector Laboratories) was used to immunostain the tissue sections with phospho-S473 Akt antibody (catalogue number 06-801-MN; Upstate Biotechnology). Endogenous peroxidase and biotin were blocked, and sections were incubated 1 h at room temperature with a 1:250 dilution of antibody to phospho-S473 Akt. The remainder of the staining procedure was performed according to the manufacturer's instructions using

diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining. Primary antibody was replaced with an equal concentration of nonimmune sheep IgG on negative control sections.

PI3K Assay. PI3K was immunoprecipitated from the tumor tissue lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), and finally with 10 mM Tris, 100 mM NaCl, 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM MgCl $_2$, 50 μM ATP, 20 μCi [γ - ^{32}P]ATP, and 10 μg of L- α -phosphatidylinositol-4,5-bisphosphate (PI-4,5-P $_2$; BIOMOL) for 20 min at 25°C . The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of CHCl $_3$ /methanol. Phosphorylated products were separated by TLC as described previously (21). The conversion of PI-4,5-P $_2$ to PI-3,4,5-P $_3$ was determined by autoradiography and quantitated by using a Phosphorimager. Average readings of the kinase activity 3-fold higher than that in normal ovarian tissue was considered as elevated PI3K activity.

Expression Constructs and GST Fusion Protein. HA epitope-tagged constitutively active, wild-type, and dominant-negative AKT2 were prepared as described previously (21). The p110 α and p85 α of PI3K expression constructs were gifts from Dr. Julian Downward (Imperial Cancer Research Fund, London, United Kingdom). The mammalian expression construct of ER α -S167A was kindly provided by Dr. Benita S. Katzenellenbogen (University of Illinois, Urbana, IL). The GST-ER α and GST-ER α -S167A were created by PCR and inserted into pcDNA3 and pEGX-4T (Pharmacia) vectors, respectively. GST-ER α fusion proteins were purified as described previously (21).

In Vivo [^{32}P] Cell Labeling. Transfected COS7 and nontransfected MCF7 cells were labeled with [^{32}P]P $_i$ (0.5 mCi/ml) in MEM without phosphate, serum, and phenol red for 4 h and lysed. ER α was immunoprecipitated with monoclonal anti-ER α or anti-myc antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated ER α was detected by autoradiography and quantitated by using Molecular Dynamics Phosphorimager with ImageQuant software.

Reporter Assay. HEK293 and MCF7 cells (8×10^5) were seeded in a 60-mm plate. The cells were cotransfected with the luciferase reporter plasmid (2ERE-MpG12), wild-type, constitutively active, or dominant-negative AKT2 and ER α , as well as pCMV- β gal plasmid as an internal control. The amount of DNA in each transfection was kept constant by the addition of empty pcDNA3 vector. Luciferase and β -galactosidase activities were determined 48 h after transfection according to the manufacturer's procedure (Promega). Luciferase activity was corrected for transfection efficiency by using the control β -galactosidase activity. All of the experiments were performed in triplicate from independent cell cultures.

Results and Discussion

Frequent Activation of AKT2 in Breast Carcinoma. We have demonstrated previously (15, 20) that AKT2, like AKT1, is activated by a number of mitogenic growth factors in a PI3K-dependent manner and that AKT2 kinase activity is frequently elevated in human ovarian tumors. To examine whether AKT2 is activated in human primary breast cancer, we performed *in vitro* kinase assays in 80 human breast carcinoma specimens, including 58 ductal infiltrating adenocarcinomas, 16 lobular carcinomas, and six mixed tumors. Lysates from tumor specimens were incubated with anti-AKT2 antibody, which specifically reacts with AKT2 (20). The immunoprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate. The results revealed an elevated level of AKT2 kinase in 32 of specimens (40%), including 29 cases with ductal infiltrating carcinoma, two lobular, and one mixed tumor (Fig. 1A). To further demonstrate AKT2 activation in breast cancer, we performed Western blot analyses of tumor lysates with phospho-Ser-473 antibody, a phosphorylation site that is critical for activation of three isoforms of Akt (17). To avoid the cross-reaction, the tumor lysates were incubated with anti-AKT2 antibody. The AKT2 immunoprecipitates were separated by SDS-PAGE and probed with phospho-Ser473 antibody. Phosphorylated AKT2 was detected only in breast tumors with elevated AKT2 kinase

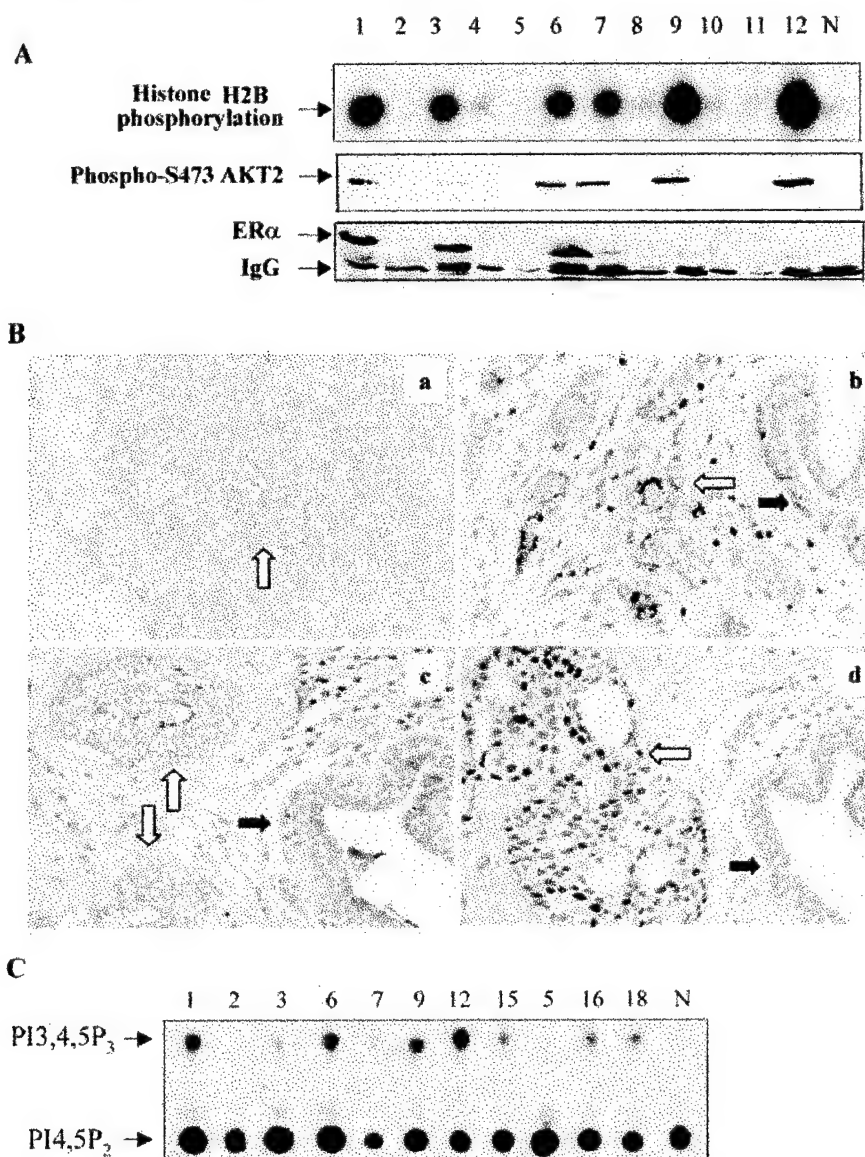


Fig. 1. Activation of AKT2 in human primary breast cancers. *A* (top panel), *in vitro* kinase assays of immunoprecipitated AKT2 from representative frozen breast tumor specimens. Normal mammary tissue (*N*) was used as a control. *Bottom panels*, Western blot analyses of AKT2 and ER α immunoprecipitates with anti-phospho-Ser473 Akt and anti-ER α antibodies, respectively. *B*, immunohistochemical staining of the paraffin sections prepared from primary breast adenocarcinomas with anti-phospho-S473 Akt (*a-c*) and anti-ER α (*d*) antibodies. Strong staining with both antibodies was observed in tumor cells (white arrows), whereas weak immunoreaction was detected in stromal tissue and adjacent ductal epithelium (black arrows). Photomicrographs *c* and *d* are the same specimen but different sections. *C*, *In vitro* PI3K assay of anti-p85 immunoprecipitates from 11 tumor and one normal specimen. The specimen numbers correspond to the same tumors shown in *A*.

activity (Fig. 1*A*). Because stromal tissues account for approximately 20–30% of the tumor specimens used in this study, we examined whether the activation of AKT2 is derived from the tumor cells or the stromal tissues by immunostaining paraffin sections with a phospho-Ser473 Akt antibody. Positive staining of tumor cells was detected in all of the 32 cases with AKT2 activation, whereas no staining was observed in normal ductal epithelial cells (Fig. 1*B*). These data suggest that activation of AKT2 is a common occurrence in human breast cancer.

Because AKT2 is a downstream target of PI3K, which is activated in colon and ovarian carcinoma (20, 22, 23), we next examined the PI3K activity in breast tumors by *in vitro* PI3K assay. Because of the fact that all of the tumors with elevated PI3K activity result in activation of Akt (20, 22–24), immunoprecipitation with a pan-p85 antibody was performed in 58 breast tumor specimens, including 32 with AKT2 activation and, as control, 26 without AKT2 activation. The ability to convert PI-4,5-P₂ to PI-3,4,5-P₃ was determined. Elevated PI3K activity was detected in all of the 32 specimens that exhibited AKT2 activation. No PI3K activation was observed in 26

specimens without AKT2 activation (Fig. 1*C*), indicating that activation of AKT2 in breast cancer predominantly results from PI3K activation. Moreover, Western blot and immunohistochemistry analyses with anti-ER α antibody revealed that 88% of the cases (28 of 32) with PI3K/AKT2 activation showed strong ER α positive (Fig. 1, *A* and *B*), whereas only 54% of the cases (14 of 26) without PI3K/AKT2 activation exhibited positive ER α , suggesting that activated PI3K/AKT2 could be involved in the regulation of ER α activity in breast cancer cells. In addition, the majority of cases with AKT2 activation are late stage (23 of 32 at stages III and IV) and poorly differentiated tumors (19 of 32), indicating that PI3K/AKT2 activation in breast cancer may be associated with tumor progression rather than initiation.

AKT2 Activates ER α -mediated Transcription in a Ligand-independent Manner. Previous studies (1, 25) have shown that MAPK is activated in breast cancer and contributes to estrogen-independent breast tumor cell growth by direct phosphorylation of ER α . Moreover, several other signal molecules, including protein kinase A, casein kinase II, pp90^{rsk1}, and MEK1/p38, have been

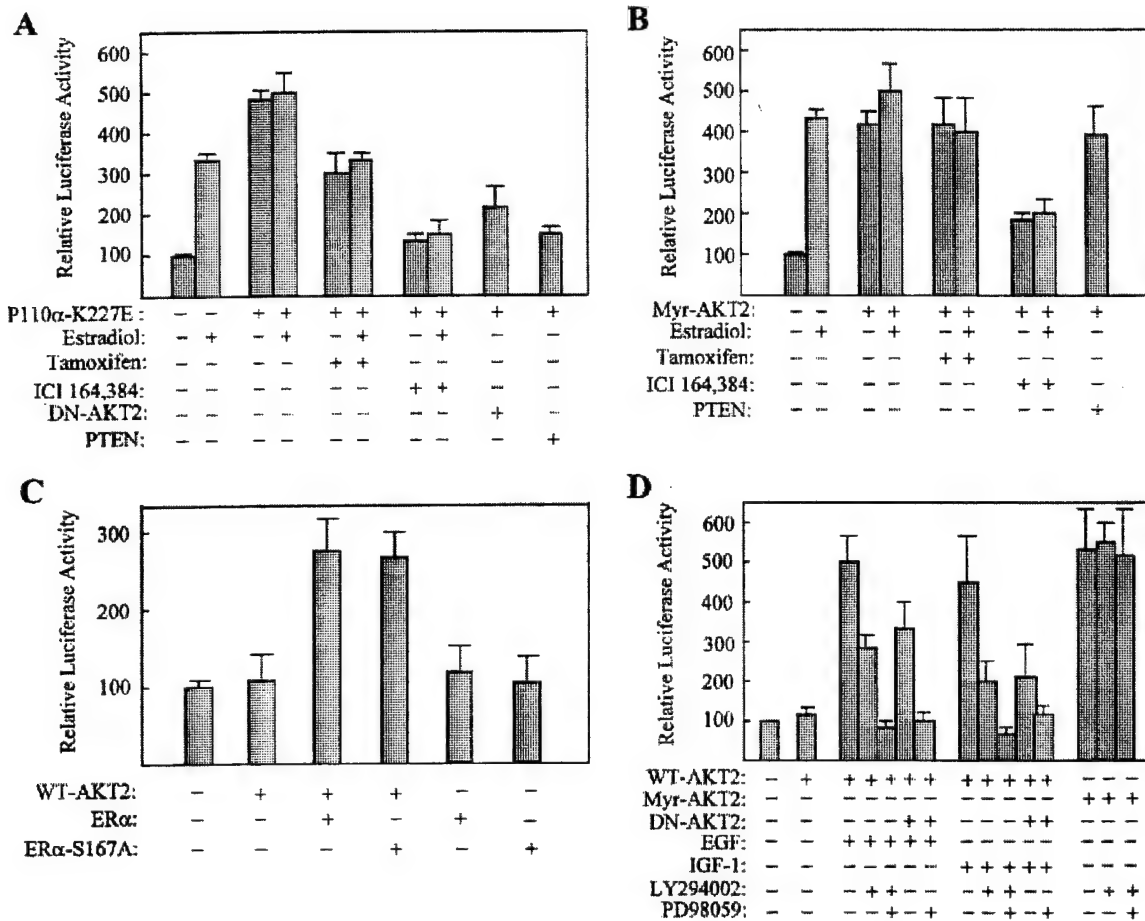


Fig. 2. AKT2 and PI3K activate ER α transcriptional activity. A–D, reporter assays: MCF-7 cells were transfected with ERE2-TK-LUC reporter, β -galactosidase, and indicated expression constructs. After 36 h of transfection, the cells were serum-starved overnight and treated with indicated agents. Luciferase activity was normalized to β -galactosidase activity.

shown to activate ER α -mediated transcription, possibly resulting in hormone-independent tumor cell growth (1, 8–10, 26). Because AKT2 and PI3K are frequently activated in breast cancer and the majority of cases with AKT2 activation are ER α positive, we investigated whether AKT2 and PI3K regulate ER α -mediated transcription. ER α -positive MCF7 breast cancer cells were transiently transfected with a reporter construct containing a luciferase gene regulated by two estrogen response elements (ERE2-TK-LUC) and a plasmid expressing β -galactosidase that allows the luciferase data to be normalized for transfection efficiency. In addition, the cells were transfected with expression constructs for constitutively activated p110 α (p110 α -K227E) subunit of PI3K, wild-type, constitutively activated, and dominant-negative AKT2 or vector alone. As shown in Fig. 2, p110 α -K227E or myr-AKT2 increased ERE2-TK-LUC activity 3–4-fold in the absence of estradiol. Constitutively activated p110 α -induced reporter activity was attenuated by dominant-negative mutant AKT2 (Fig. 2A). Tamoxifen (4-hydroxytamoxifen), an antiestrogen reagent that inhibits transcriptional activation by AF2 but not through AF1 (5), abolished estradiol-enhanced transcription but had no effects on p110 α -K227E and myr-AKT2-stimulated ER α activity (Fig. 2, A and B), suggesting that PI3K/AKT2-increased ER α transcriptional activity is regulated by phosphorylation of ER α within the AF1 region and could be involved in tamoxifen resistance.

ICI 164,384, which causes rapid degradation of ER α (6, 27), completely blocked PI3K- and AKT2-induced reporter activity. PTEN, a tumor suppressor encoding a lipid phosphatase that nega-

tively regulates PI3K, inhibited constitutively active p110-induced ER α -mediated transcription but had no effect on constitutively activated AKT2-stimulated ER α activity (Fig. 2B).

Moreover, we have observed that exogenous expression of ER α in ER α -positive MCF7 cells increased wild-type AKT2-induced ERE2-TK-LUC activity 2–3-fold as compared with cells transfected with wild-type AKT2 alone (Fig. 2C), implying that ER α might activate AKT2 kinase and subsequently enhance its own transcriptional activity (see below). Taken collectively, these data indicate that PI3K/AKT2-activated ER α -mediated transcription is estrogen-independent and that the frequently elevated level of PI3K/AKT2 kinase in primary breast cancer could relate the refractoriness of hormone therapy.

AKT2 Mediates Growth Factor-induced ER α Transcriptional Activity. A very recent study (28) showed that Akt1 mediates the estrogenic functions of EGF and IGF1. Next, we examined the possible role of AKT2 in growth factor-induced ER α activation. ER α -positive MCF7 cells were transfected with ERE2-TK-LUC and dominant-negative, wild-type, or constitutively activated AKT2 or vector alone and were treated with or without either 100 ng/ml EGF or 50 ng/ml IGF1 (Fig. 2D). Treatment with the growth factors resulted in an approximately 4.5-fold increase in ER α -mediated transcriptional activity. The EGF- and IGF1-induced reporter activity was partially abrogated by dominant-negative AKT2 or PI3K inhibitor LY294002 and completely blocked by the combination of PI3K and MAPK inhibitors (LY294002 and PD98059). However, the combined inhibitors had no effect on constitutively activated AKT2-induced reporter

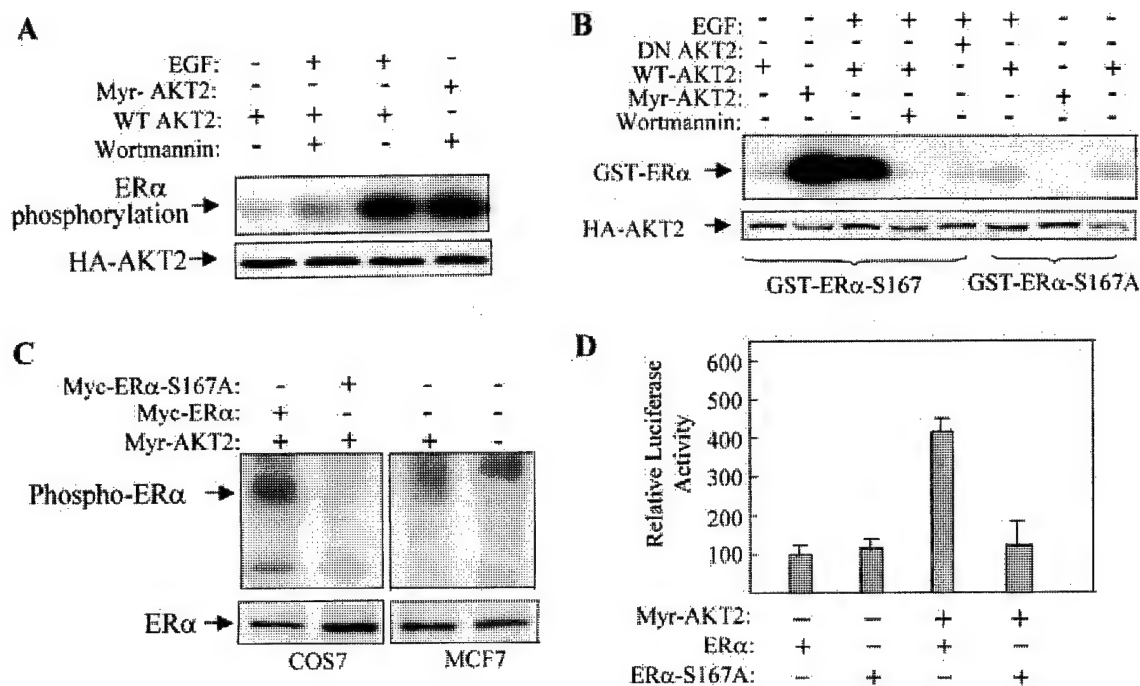


Fig. 3. AKT2 phosphorylates ER α on serine-167 *in vitro* and *in vivo*. *In vitro* AKT2 kinase assay of the immunoprecipitates from HEK293 cells transfected with indicated expression constructs. Full length of human recombinant ER α (A), GST-ER α -S167, and GST-ER α -S167A (B) were used as substrates. C, COS7 and MCF7 cells were transfected with indicated plasmids and incubated with [32 P]P $_i$ for 4 h. Immunoprecipitates were prepared with anti-myc (left) or anti-ER α (right) antibody and separated by SDS-PAGE. After transfer, the membrane was exposed to a film (top) and detected with anti-ER α antibody (bottom). D, AKT2 phosphorylation of serine-167 is essential for AKT2-induced ER α transcriptional activity. Luciferase reporter assay of HEK293 cells transfected with ERE2-TK-LUC, wild-type ER α , or ER α -S167A, β -galactosidase, and myr-AKT2.

activity. These results suggest that the "steroid-independent activation" of ER α by growth factors is mediated by the PI3K/AKT2 pathway, in addition to MAPK, PKA, casein kinase II, and pp90^{rsk1}.

AKT2 Phosphorylates Serine-167 of ER α *in Vitro* and *in Vivo*. Phosphorylation of ER α has been shown to be an important mechanism by which ER α activity is regulated. ER α is hyperphosphorylated on multiple sites in response to hormone binding and growth factor stimulation (1–4). Transcriptional activation by growth factors has been shown to require AF-1 but not AF-2 (1, 8–10). There is evidence to suggest that EGF and IGF-1 induce MAPK and pp90^{rsk1}/casein kinase II activity leading to phosphorylation of serine-118 and serine-167, respectively, in AF-1 region (4, 5, 8–10). To examine whether AKT2 phosphorylates ER α *in vitro*, HEK293 cells were transfected with HA-tagged wild-type and constitutively activated AKT2, and immunoprecipitation was prepared with anti-HA antibody. *In vitro* AKT2 kinase assays, using full length of human recombinant ER α as substrate, revealed that constitutively activated AKT2 and EGF-induced AKT2 strongly phosphorylated hER α . The ER α phosphorylation that was induced by EGF-stimulated AKT2 was abrogated by wortmannin (Fig. 3A).

To determine whether AKT2 phosphorylates ER α *in vivo*, MCF7 cells were transfected with constitutively activated AKT2 or pcDNA3 vector alone and labeled with [32 P]P $_i$. The cell lysates were incubated with anti-ER α antibody, and the immunoprecipitates were separated on SDS-PAGE. ER α was highly phosphorylated in constitutively activated AKT2-transfected cells but in the cells transfected with vector alone (Fig. 3C). These data indicate that AKT2 phosphorylated ER α both *in vitro* and *in vivo*.

Martin *et al.* (28) recently demonstrated that EGF- and IGF1-induced Akt1 potentiates the AF-1 function of ER α , possibly through the phosphorylation of serine residues. There are four serine residues

(Ser-104, Ser-106, Ser-118, and Ser-167) in the AF-1 region of the receptor that are predominantly phosphorylated in response to estrogen and growth factor stimulation (1–4). We examined the ER α protein sequence and found that serine-167 (¹⁶²ERLAS¹⁶⁷) is a putative AKT2 phosphorylation site. Constructs expressing GST-fused wild-type and mutant (S167A) AF-1 region were created. *In vitro* kinase assays revealed that myr-AKT2 and EGF-stimulated AKT2 strongly phosphorylated GST-ER α -S167 but not GST-ER α -S167A mutant (Fig. 3B). The EGF-induced AKT2 phosphorylation of ER α is blocked by wortmannin. To examine whether AKT2 phosphorylates serine-167 *in vivo*, COS7 cells were transfected with myc-tagged wild-type and mutant (S167A) human ER α expression constructs together with constitutively activated AKT2. After 36 h of transfection, the cells were incubated with [32 P]P $_i$ and immunoprecipitated with anti-myc antibody. As demonstrated in Fig. 3C, constitutively active AKT2 phosphorylated wild-type ER α but not the ER α -S167A mutant *in vivo*, suggesting that serine-167 of ER α is a physiological substrate for AKT2.

Previous studies (29) showed that serine-167 is important for ER α transcriptional activity. To further examine whether AKT2-activated ER α transcriptional activity depends upon phosphorylation of serine-167, reporter assays were carried out in HEK293 cells transfected with ERE2-TK-LUC, constitutively activated AKT2, and ER α -S167A or wild-type ER α . Fig. 3D shows that ER α -S167A had no ability to mediate constitutively activated AKT2-induced ERE2-TK-LUC reporter activity, indicating that AKT2 regulates ER α -mediated transcription through phosphorylation of serine-167.

ER α Binds To and Activates PI3K/AKT2 in Epithelial Cells via a Ligand-independent Mechanism. Recent studies (30, 31) demonstrated that ER α binds to the p85 α regulatory subunit of PI3K after estradiol treatment, leading to the activation of PI3K/Akt and endo-

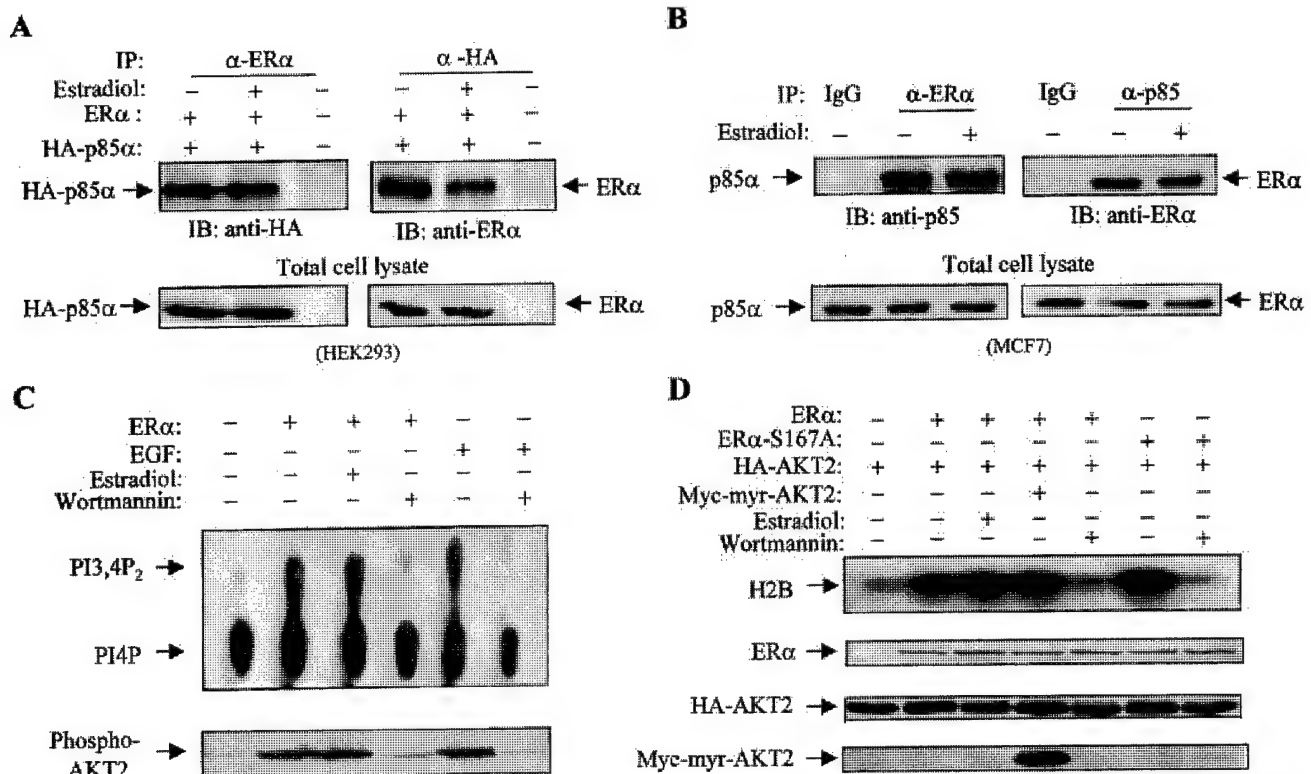


Fig. 4. ER α interacts with p85 α and activates the PI3K/AKT2 pathway in human epithelial cells. Coimmunoprecipitation of ER α and p85 α in (A) HEK293 cells cotransfected with HA-p85 α /ER α and in (B) nontransfected MCF7 cells. Top, coimmunoprecipitation; bottom, Western blot of total cell lysates. C, *in vitro* PI3K assay (top) of HEK293 cells transfected and treated with indicated plasmid and agents. Bottom, Western blotting analysis of AKT2 immunoprecipitates with phospho-S473 Akt antibody. D, *in vitro* kinase assay (top) of HA-AKT2 immunoprecipitates prepared from HEK293 cells transfected with indicated expression constructs, using histone H2B as a substrate. Panels 2–4, Western blots of transfected HEK293 cell lysates detected with anti-ER α , anti-HA, or anti-myc antibody.

thelial nitric oxide synthase in endothelial cells. In the absence of estradiol, ER α failed to bind and activate PI3K, indicating that ER α -associated PI3K in endothelial cells is estrogen-dependent (30). Next, we examined whether ER α binds to and activates PI3K/AKT2 in epithelial cells. ER α /HA-p85 α -transfected HEK293 and nontransfected ER α -positive MCF7 cells were immunoprecipitated with anti-ER α and detected with anti-HA or anti-p85 α antibody or *vice versa*. As shown in Fig. 4A and B, ER α constitutively associated with p85 α , and this interaction was unaffected by estradiol treatment. In addition, *in vitro* PI3K assays revealed that expression of ER α in HEK 293 cells significantly induced PI3K activity in the absence or presence of estradiol (Fig. 4C). These data suggest that ER α binding to and activating PI3K is ligand-independent in epithelial cells.

Next, we examined whether ER α activates AKT2 and whether this activation is dependent on AKT2 phosphorylation. ER α -negative HEK293 cells were transfected with ER α or ER α -S167A, together with HA-AKT2. *In vitro* AKT2 kinase assays revealed that ER α significantly activates AKT2 in the absence of estradiol. Additional estradiol treatment did not further enhance ER α -induced AKT2 activation. The PI3K inhibitor, wortmannin, completely abolished the activation. Interestingly, ER α -S167A activated AKT2 at a similar level to that of wild-type ER α . Coexpression of myc-tagged constitutively active AKT2 (Myc-myr-AKT2) and ER α had the same effect on wild-type AKT2 activation as that of expression of ER α alone (Fig. 4D). These results indicate that activation of AKT2 by ER α is through PI3K and independent of ER α phosphorylation by PI3K/AKT2.

In summary, we demonstrate in this study that AKT2 and PI3K are frequently activated in primary human breast carcinoma. The PI3K/AKT2 pathway regulates ER α transcriptional activity by phosphoryl-

ation of serine-167 *in vitro* and *in vivo*, and ER α activates PI3K/AKT2 kinase by binding to p85 α in a ligand-independent manner in epithelial cells. This study suggests that the PI3K/AKT2 pathway may play a pivotal role in estrogen-independent breast cancer cell growth and tamoxifen-resistance; therefore, it could represent an important therapeutic target in human breast cancer.

Acknowledgments

We thank Tissue Procurement, DNA Sequence, and Tissue Pathology Core Facilities at the H. Lee Moffitt Cancer Center for providing cancer specimens, sequencing, and immunohistochemical staining. We also thank Kun Jiang and Geng Wang for technical support. Mei Sun is a Research Fellow from Beijing Scientific Association, Beijing, China.

References

- Nicholson, R. I., McClelland, R. A., Robertson, J. F., and Gee, J. M. Involvement of steroid hormone and growth factor cross-talk in endocrine response in breast cancer. *Endocr. Relat. Cancer*, 6: 373–387, 1999.
- Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. The nuclear receptor superfamily: the second decade. *Cell*, 83: 835–839, 1995.
- Weigel, N. L. Steroid hormone receptors and their regulation by phosphorylation. *Biochem. J.*, 319: 657–667, 1996.
- Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science (Wash. DC)*, 270: 1491–1494, 1995.
- Berry, M., Metzger, D., and Chambon, P. Role of the two activating domains of the estrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-estrogen 4-hydroxytamoxifen. *EMBO J.*, 9: 2811–2818, 1990.
- McDonnell, D. P., Clemm, D. L., Hermann, T., Goldman, M. E., and Pike, J. W. Analysis of estrogen receptor function *in vitro* reveals three distinct classes of antiestrogens. *Mol. Endocrinol.*, 9: 659–669, 1995.

7. Katzenellenbogen, B. S., Montano, M. M., Ekena, K., Herman, M. E., and McInerney, E. M. Antiestrogens: mechanisms of action and resistance in breast cancer. *Breast Cancer Res. Treat.*, **44**: 23–38, 1997.
8. Joel, P. B., Smith, J., Sturgill, T. W., Fisher, T. L., Blenis, J., and Lannigan, D. A. pp90^{rtk} regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol. Cell. Biol.*, **18**: 1978–1984, 1998.
9. Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. Phosphorylation of the human estrogen receptor by mitogen-activated protein kinase and casein kinase II: consequence on DNA binding. *J. Steroid Biochem. Mol. Biol.*, **55**: 163–172, 1995.
10. Chen, D., Pace, P. E., Coombes, R. C., and Ali, S. Phosphorylation of human estrogen receptor α by protein kinase A regulates dimerization. *Mol. Cell. Biol.*, **19**: 1002–1015, 1999.
11. Kelly, M. J., and Levin, E. R. Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol. Metab.*, **12**: 152–156, 2001.
12. Razandi, M., Pedram, A., and Levin, E. R. Estrogen signals to the preservation of endothelial cell form and function. *J. Biol. Chem.*, **275**: 38540–38546, 2000.
13. Kahlert, S., Nuedling, S., van Eickels, M., Vetter, H., Meyer, R., and Grohe, C. Estrogen receptor α rapidly activates the IGF-1 receptor pathway. *J. Biol. Chem.*, **275**: 18447–18453, 2000.
14. Franke, T. F., Yang, S. L., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. The protein kinase encoded by the *Akt* proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*, **81**: 727–736, 1995.
15. Liu, A.-X., Testa, J. R., Hamilton, T. C., Jove, R., Nicosia, S. V., and Cheng, J. Q. AKT2, a member of the protein kinase B family, is activated by growth factors, v-Ha-ras, and v-src through phosphatidylinositol 3-kinase in human ovarian epithelial cancer cells. *Cancer Res.*, **58**: 2973–2977, 1998.
16. Nakatani, K., Sakaue, H., Thompson, D. A., Weigel, R. J., and Roth, R. A. Identification of a human Akt3 (protein kinase B γ) which contains the regulatory serine phosphorylation site. *Biochem. Biophys. Res. Commun.*, **257**: 906–910, 1999.
17. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.*, **68**: 965–1014, 1999.
18. Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamilton, T. C., Tsichlis, P. N., and Testa, J. R. *AKT2*, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc. Natl. Acad. Sci. USA*, **89**: 9267–9271, 1992.
19. Cheng, J. Q., Ruggeri, B., Klein, W. M., Sonoda, G., Altomare, D. A., Watson, D. K., and Testa, J. R. Amplification of *AKT2* in human pancreatic cancer cells and inhibition of *AKT2* expression and tumorigenicity by antisense RNA. *Proc. Natl. Acad. Sci. USA*, **93**: 3636–3641, 1996.
20. Yuan, Z., Sun, M., Feldman, R. I., Wang, G., Ma, X., Coppola, D., Nicosia, S. V., and Cheng, J. Q. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene*, **19**: 2324–2330, 2000.
21. Jiang, K., Coppola, D., Crespo, N. C., Nicosia, S. V., Hamilton, A. D., Sebt, S. M., and Cheng, J. Q. The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. *Mol. Cell. Biol.*, **20**: 139–148, 2000.
22. Benistant, C., Chapuis, H., and Roche, S. A specific function for phosphatidylinositol 3-kinase α (p85 α -p110 α) in cell survival and for phosphatidylinositol 3-kinase β (p85 α -p110 β) in *de novo* DNA synthesis of human colon carcinoma cells. *Oncogene*, **19**: 5083–5090, 2000.
23. Shayesteh, L., Lu, Y., Kuo, W. L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G. B., and Gray, J. W. PIK3CA is implicated as an oncogene in ovarian cancer. *Nat. Genet.*, **21**: 99–102, 1999.
24. Bai, R. Y., Ouyang, T., Miething, C., Morris, S. W., Peschel, C., and Duyster, J. Nucleophosmin-anaplastic lymphoma kinase associated with anaplastic large-cell lymphoma activates the phosphatidylinositol 3-kinase/Akt antiapoptotic signaling pathway. *Blood*, **96**: 4319–4327, 2000.
25. Coutts, A. S., and Murphy, L. C. Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. *Cancer Res.*, **58**: 4071–4074, 1998.
26. Lee, H., Jiang, F., Wang, Q., Nicosia, S. V., Yang, J., Su, B., and Bai, W. MEKK1 activation of human estrogen receptor α and stimulation of the agonistic activity of 4-hydroxytamoxifen in endometrial and ovarian cancer cells. *Mol. Endocrinol.*, **14**: 1882–1896, 2000.
27. Simard, J., Sanchez, R., Poirier, D., Gauthier, S., Singh, S. M., Merand, Y., Belanger, A., Labrie, C., and Labrie, F. Blockade of the stimulatory effect of estrogens, OH-tamoxifen, OH-toremifene, droloxifene, and raloxifene on alkaline phosphatase activity by the antiestrogen EM-800 in human endometrial adenocarcinoma Ishikawa cells. *Cancer Res.*, **57**: 3494–3497, 1997.
28. Martin, M. B., Franke, T. F., Stoica, G. E., Chambon, P., Katzenellenbogen, B. S., Stoica, B. A., McLemore, M. S., Olivo, S. E., and Stoica, A. A role for Akt in mediating the estrogenic functions of epidermal growth factor and insulin-like growth factor I. *Endocrinology*, **141**: 4503–4511, 2000.
29. Castano, E., Vorojeikina, D. P., and Notides, A. C. Phosphorylation of serine-167 on the human estrogen receptor is important for estrogen response element binding and transcriptional activation. *Biochem. J.*, **326**: 149–157, 1997.
30. Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W., and Liao, J. K. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature (Lond.)*, **407**: 538–541, 2000.
31. Hisamoto, K., Ohmichi, M., Kurachi, H., Hayakawa, J., Kanda, Y., Nishio, Y., Adachi, K., Tasaka, K., Miyoshi, E., Fujiwara, N., Taniguchi, N., and Murata, Y. Estrogen induces the Akt-dependent activation of endothelial nitric oxide synthase in vascular endothelial cells. *J. Biol. Chem.*, **276**: 3459–3467, 2001.

Ultraviolet Irradiation- and Dimethyl Sulfoxide-Induced Telomerase Activity in Ovarian Epithelial Cell Lines

Michelle Y. Alfonso-De Matte, Jin Q. Cheng, and Patricia A. Kruk¹

University of South Florida and the H. Lee Moffitt Cancer Center, Tampa, Florida 33612

Information about telomerase regulation is incomplete, especially since various studies suggest complexity in telomerase regulation. Given the important association between telomerase and cancer, it is imperative to design and develop a model system in which telomerase activity can be regulated and studied. We employed ultraviolet (UV) radiation or dimethyl sulfoxide (DMSO) to transiently induce telomerase activity in a telomerase-positive cell line and, most importantly, in a telomerase-negative cell line. UV- or DMSO-induced telomerase activity was associated with increased hTERT, but not hTR, mRNA transcription in the telomerase-negative cells. However, no changes in hTERT or hTR mRNA transcription were noted with UV- or DMSO-induced telomerase activity in the telomerase-positive cells. Inhibition of protein synthesis or the phosphatidylinositol 3-kinase (PI3K) pathway suppressed telomerase induction and/or activity in all cell lines examined, suggesting telomerase activity was dependent on protein synthesis and PI3K-mediated phosphorylation. Furthermore, enhanced telomerase activity was limited to UV and DMSO, since a variety of chemotherapeutic agents failed to induce telomerase activity. Therefore, our data provide a useful culture model system to study telomerase regulation in telomerase-negative and -positive cell lines and from which to obtain information about telomerase as a target for cancer intervention. © 2001

Academic Press

Key Words: telomerase; ovarian cancer; UV irradiation; hTERT; PI 3-kinase; dimethyl sulfoxide; wortmannin; LY294002; hTR; cycloheximide.

INTRODUCTION

Telomerase is a ribonucleoprotein that synthesizes telomeric repeats *de novo*. Telomerase is composed of three subunits: an RNA component (hTR), an additional protein component (TLP1), and a catalytic com-

ponent (hTERT). While little information is available about the TLP1 component, the RNA component has been shown to be constitutively expressed in most cells [1] and serves as the template for the telomerase reverse transcriptase to lay down telomeric repeats. Studies suggest that hTERT is the rate-limiting component essential for telomerase activity and that it is transcriptionally regulated independently of hTR [2–5]. In addition, telomerase regulation appears to be complex, requiring the coordinated involvement of numerous proteins, including telomeric binding proteins TRF1 and TRF2, as well as tankyrase [6–9]. While reports correlating telomerase activity with cell cycle regulation are contradictory [10–14], several studies report abrogation of telomerase activity with differentiating agents, including dimethyl sulfoxide (DMSO) [15, 16], deoxynucleoside analogues [17], tea catechins [18], and the reverse transcriptase inhibitor AZT [19]. Molecular mechanisms of telomerase regulation include methylation of the hTERT gene promoter [20] and *c-Myc*-driven transcription of hTERT [21–23], resulting in increased telomerase activity. A recent report of hTERT protein phosphorylation by Akt kinase [24] implies a possible role for the phosphatidylinositol 3-kinase (PI3K) signal transduction pathway in the regulation of telomerase activity.

Telomerase is present in more than 90% of all tumors, including ovarian cancer, which has the highest mortality rate among gynecological cancers (5-year survival is 37%). Furthermore, telomerase activity has been correlated with tumor stage and aggressiveness in tumors of breast, head/neck, lung, gastrointestinal, pancreas, liver, prostate, kidney/bladder, brain, skin, and ovary as well as leukemic and lymphomatous tumors [25]. Since activated Akt is frequently detected in breast, prostate, and ovarian carcinomas [26], and since Akt, a downstream target of PI3K, appears to play a role in telomerase regulation, we sought to develop a controlled culture model system to study telomerase regulation in ovarian cancer. We employed ultraviolet (UV) or DMSO to transiently elevate endogenous telomerase activity in a telomerase-positive cell line and, more importantly, to transiently induce *de novo*

¹ To whom correspondence and reprint requests should be addressed at Department of Pathology, MDC 11, University of South Florida, College of Medicine, 12901 Bruce B. Downs Boulevard, Tampa, FL 33612. Fax: (813) 974-5536. E-mail: pkruk@hsc.usf.edu.



telomerase activity in a telomerase-negative cell line. UV- or DMSO-induced telomerase activity was associated with increased hTERT mRNA levels only in previously telomerase-negative cell lines. Induced telomerase activity was not related to hTERT mRNA levels, but appeared to require protein synthesis in all cell lines examined. We demonstrated an increase in PI3K activity following UV treatment [27–33]. We also showed, for the first time, that DMSO can also increase PI3K activity. Consequently, inhibition of PI3K suppressed telomerase activity, further supporting a role for PI3K-mediated regulation of telomerase activity. Therefore, not only have we developed a model for telomerase induction in a telomerase-positive cell line but, more importantly, we also were able to induce telomerase activity in a previously telomerase-negative cell line. This model system provides a useful tool for the study of telomerase regulation in order to evaluate telomerase as a potential target for cancer intervention.

METHODS AND MATERIALS

Cell culture. Two ovarian carcinoma cell lines, SW626 and CaOV3 (obtained from ATCC, Rockville, MD) were used. Although the origin of the SW626 cell line has been questioned [34], this cell line has been described as an ovarian carcinoma cell line. Four nontumorigenic, SV40 large-T-antigen-transfected ovarian surface epithelium cell lines (IOSE) derived from normal ovarian surface epithelium [35], FHIOSE 135, FHIOSE 118, NFHIOSE 80, and NFHIOSE 144, were also used in the present study. All IOSE cell lines were previously determined to be telomerase negative [36]. Cells were maintained in Medium 199/MDCB 105 (1:1) (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT) and 10 $\mu\text{g}/\text{ml}$ gentamicin (GIBCO BRL, Grand Island, NY) in a humidified 5% $\text{CO}_2/95\%$ air atmosphere.

Ultraviolet irradiation. Exponentially growing cultures of IOSE and SW626 cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS), drained, and irradiated with UV radiation at 2–40 J/m^2 using a 254-nm germicidal lamp. Altering the exposure time controlled for the total dose of radiation. Following irradiation, fresh medium was added and cells were incubated until their collection at various time intervals. Control samples were rinsed with DPBS and drained, and fresh medium was added.

Treatment with chemotherapeutic and chemical agents. To examine the effect of chemotherapeutic agents on telomerase activity, cells were treated with carboplatinum (CB), etoposide (ET), or cisplatin (CP). CB, ET, and CP were dissolved in fresh, serum-free medium. Doses used were CB, 1, 10, and 25 μM ; ET, 10, 50, and 100 μM ; and CP, 25 and 100 μM . In order to determine the effects of a differentiating agent on telomerase activity, cells were treated with DMSO. Final concentrations of DMSO ranged from 0.05 to 1.0% in serum-free medium. For treatment, cells were rinsed with DPBS and the medium containing an agent was added. Controls consisted of cells that received only serum-free medium. Following 2 h of treatment, control and experimental cells were rinsed with DPBS and fresh medium containing 5% FBS was added. Cells were then harvested at various time intervals following treatment.

Treatment with cycloheximide. To determine if new protein synthesis was required for induction of telomerase activity, SW626 and FHIOSE 118 cells were treated with cycloheximide (CHX). CHX was dissolved directly into Medium 199/MDCB 105 (1:1) for a final concentration of 40 μM . For UV-treated cells, CHX-containing medium was added to exponentially growing cells immediately following UV

treatment until the time of collection. For DMSO-treated cells, DMSO was added to the CHX-containing medium at the previously described concentrations. When the DMSO treatment period was completed, cells were rinsed twice with DPBS and fresh CHX-containing medium was added until the time of collection.

Telomerase assay. To quantitatively detect changes in telomerase levels, all cells were assayed for telomerase activity using the telomerase polymerase chain reaction–enzyme-linked immunosorbent assay (PCR–ELISA) (Roche Molecular Biochemicals, Indianapolis, IN), as described previously [36] and according to the manufacturer's instructions. This assay has been demonstrated to be as sensitive as the radioactive, telomere repeat amplification protocol (TRAP) assay [37–44]. Briefly, cells were washed with DPBS, trypsinized, counted, and centrifuged at 500g for 5 min. Pellets were washed twice in DPBS, then resuspended in 200 μl of lysis buffer and kept on ice for 30 min, after which time the lysates were centrifuged at 100,000g for 60 min at 4°C. Lysates were then assayed using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) for the determination of protein concentration following detergent solubilization, according to the manufacturer's instructions. In order to perform the telomerase PCR–ELISA within a linear range, IOSE and SW626 cell extracts equivalent to 2 μg of protein were used. Due to its high level of telomerase activity, the volume of cell extract used for the CaOV3 cell line, which served as a positive control, was equivalent to 1 μg of protein. Following PCR–ELISA, telomerase activity was detected using a Dynex-MRX plate reader (Dynex Technologies, Chantilly, VA) and recorded as absorbance units. These values were expressed as a fold increase above control levels, with the control value used as the denominator for the determination of fold increase for the treated samples. Telomerase activity is shown $\pm\text{SE}$ if the experiments were repeated in triplicate.

Treatment with inhibitors of PI3K. SW626 and FHIOSE 118 cells were treated with wortmannin (WM) and/or LY294002 (LY), potent and specific inhibitors of PI3K [45–48], to determine if the PI3K signal transduction pathway was involved in the regulation of telomerase. For these experiments, all cells were initially serum-starved for 12 h. Following serum starvation, cells were treated with PI3K inhibitors, 100 nM WM or 10 μM LY, in serum-free medium for 12 h while parallel cultures were maintained in serum-free medium. When WM was used, the medium was replaced with fresh serum-free, WM-containing medium every 90 min due to the short half-life of WM. Cells were then UV-irradiated at 30 J/m^2 or treated with 0.5% DMSO as described earlier, or remained untreated. Triplicate samples of SW626 and FHIOSE 118 cells were collected at 8 and 24 h, respectively, and assayed for telomerase activity. In addition, RNA was isolated from SW626 and FHIOSE 118 cells at 4, 8, or 16 h following treatment to determine the levels of hTERT and hTERT mRNA by reverse transcription polymerase chain reaction (RT–PCR).

PI 3-kinase assay. In order to determine if PI3K was involved in transduction of the signal that regulates telomerase activity, a PI 3-kinase assay was performed [49, 50]. SW626 and FHIOSE 118 cells were treated with UV or DMSO, as described above. Thirty minutes following treatment, cells were rinsed once with cold DPBS and lysed with a protein lysis buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1 mM MgCl_2 , 1 mM CaCl_2 , 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM aprotinin, 2 μM leupeptin, 20 mM NaF, 10 mM $\text{Na}_2\text{P}_2\text{O}_7$, 1 mM Na_2VO_4 , 25 mM $\beta\text{-GP}$, 1 mM DTT, 2 mM benzamide). The lysates were first assayed for protein concentration, as described previously, to ensure equal amounts of protein per sample. The lysates were then briefly vortexed and centrifuged for 5 min at 12,000g and 4°C. To preclear the protein samples, supernatants were transferred to fresh Eppendorf tubes containing 25 μl protein A:protein G Sepharose beads (2:1) and 3 μl rabbit serum and then incubated at 4°C for 20 min. Samples were then centrifuged for 1 min at 6000g and 4°C. Supernatants were transferred to fresh Eppendorf tubes containing 4 μg of anti-p110 α (Upstate Biotechnology, Lake Placid, NY) and incubated for 1 h at 4°C. Thirty microliters

of Protein A Sepharose beads was added to each sample, followed by a second 1 h-incubation at 4°C. Samples were then centrifuged for 1 min at 5800g and 4°C. Supernatants were discarded and beads were washed once with 1 ml of buffer 1 (DPBS and 1 mM PMSF, 1 mM DTT, 0.1 mM Na₃VO₄), twice with 1 ml of buffer 2 (0.5 M LiCl, 0.1 M Tris, pH 7.4, 1 mM PMSF, 1 mM DTT, 0.1 mM Na₃VO₄), and once with 1 ml of buffer 3 (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 0.1 mM Na₃VO₄). Between washes, samples were centrifuged for 1 min at 5800g and 4°C. The PI-4,5-P₂ substrate (Sigma) was dissolved in chloroform (1 mg/ml). Ten microliters of substrate solution was then vacuum dried for 3 min, redissolved with 25 μ l of 20 mM Hepes at pH 7.4, and sonicated in a water bath for 20 min. This substrate solution was then added to the washed beads and incubated on ice for 20 min. Twenty-five microliters of the final reaction mixture (5 mM MgCl₂, 50 μ M ATP, 20 μ Ci [γ -³²P]ATP) was then added to each sample and incubated at room temperature for 20 min. The reaction was terminated with 100 μ l 1 M HCL. The phospholipids were then extracted with 200 μ l of CHCl₃:MeOH (1:1), and the organic phase was washed once with 100 μ l of MeOH:1M HCl (1:1). The organic phase was then vacuum dried, redissolved in 20 μ l of CHCl₃:MeOH (1:1), and spotted onto a silica gel plate (pretreated with 1% potassium oxalate and baked at 50°C for 1 h). Phosphorylated lipids were separated with CHCl₃:MeOH:4 M NH₄OH (9:7:2), visualized with a Storm Phosphorimager, and analyzed using ImageQuant software. The results are described as fold increase.

RT-PCR. To examine the contribution of transcriptional control in telomerase regulation, RT-PCR studies were performed, with each experiment repeated a minimum of three separate times. Total RNA was collected using TRizol reagent (GIBCO BRL). One microgram total RNA, oligo(dT), and reverse transcriptase were used to generate single-strand cDNA for each sample, as previously described [51]. To insure there was no DNA contamination, each sample for reverse transcription was prepared in duplicate, with the duplicate preparation lacking reverse transcriptase [52]. The cDNA samples were amplified using the Perkin-Elmer (Palo Alto, CA) GeneAmp kit. The hTERT primers used were hTERT-S (CGGAAGAGTGTCTGGAGCCAA) and hTERT-AS (GGATGAAGCGGAGTCTGGA) oligonucleotides (Sigma Genosys, The Woodlands, TX) with β -actin primers actin-S (CAGGTCAT-CACCATTGGCAATGAGC) and actin-AS (GATGTCCACGTCA-CACTTCATGA) for an internal control. PCR was performed for 33 cycles of 95°C for 20 s, 68°C for 40 s, and 72°C for 30 s. β -Actin primers were added at cycle 16. For hTR, the primers used were hTR-46S (CTAACCTAACTGAGAAGGGCGTAG) and hTR-148AS (GAAGGCGGCAGGCCGAGGCTTTTCC). PCR was performed for 28 cycles of 95°C for 20 s, 68°C for 40 s, and 72°C for 30 s. β -Actin primers were added at cycle 13 [51]. The amplified products were then separated by electrophoresis on a 9% polyacrylamide gel, stained with 1 \times SyberGreen (FMC Bioproducts, Rockland, ME), and analyzed with the Kodak EDAS 120 Digital Analysis System. Net hTERT and hTR mRNA intensities from treated samples were normalized to their corresponding β -actin mRNA levels and were expressed as a percentage of the control samples that were similarly normalized to their corresponding β -actin mRNA levels. For graphical representation, SW626 controls were set at 100%, while 118 controls (telomerase negative) were set at 0%.

Statistical analysis. Samples for telomerase PCR-ELISA were run in triplicate and the data subjected to Student *t* test analysis for determination of statistical significance for telomerase induction or suppression between treated and untreated samples. The standard error was shown only if the experiment was repeated in triplicate.

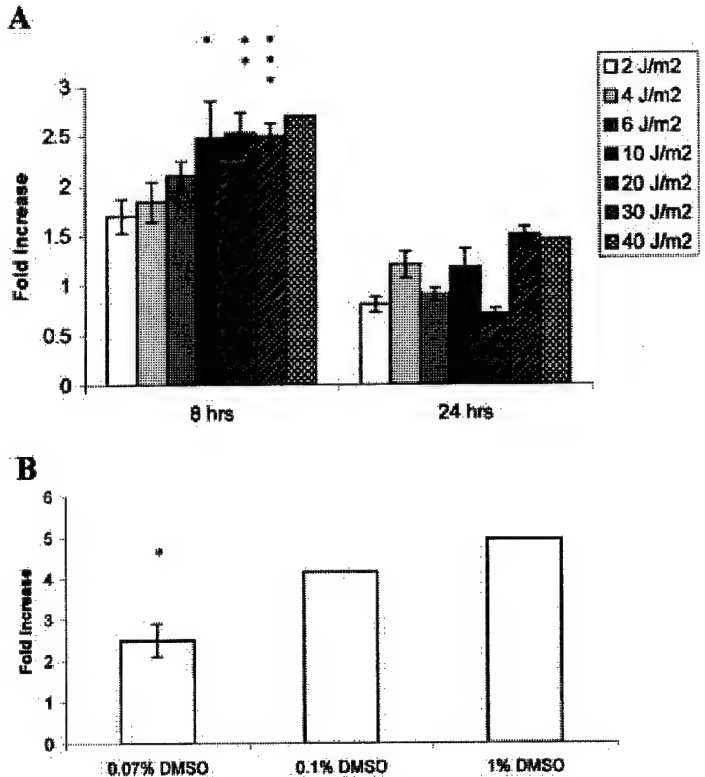


FIG. 1. UV- and DMSO-induced telomerase activity in the SW626 cell line. By using absorbance values of the control samples as the denominator, fold increase of the treated samples was determined. (A) UV-induced telomerase activity was measured at 0, 8, and 24 h, following 2, 4, 6, 10, 20, 30, and 40 J/m² of UV in triplicate samples of SW626 cells by PCR-ELISA, and the results are expressed as fold increase \pm SE. Treatment with 40 J/m² was performed only in duplicate. **P* \leq 0.04, ***P* \leq 0.03, ****P* \leq 0.03. (B) DMSO-induced telomerase activity in SW626 cells measured 8 h following 0.07, 0.1, and 1.0% DMSO. Triplicate samples were assayed for telomerase activity and the results expressed as fold increase \pm SE, **P* \leq 0.005.

RESULTS

UV and DMSO elevate basal telomerase activity and induce de novo telomerase activity. For each cell line employed, sublethal doses of UV were determined using a cell proliferation assay following various doses of UV (data not shown). With the control absorbance value set as 1 (not shown), a peak 2.75-fold elevation in endogenous telomerase activity was observed 8 h following treatment of SW626 cancer cells with 2–40 J/m² (Fig. 1A). We found that telomerase induction was related to dose, with telomerase activity increasing linearly between 2 and 10 J/m², then plateauing between 10 and 40 J/m², with almost a 3-fold increase following treatment with 40 J/m² (Fig. 1A). Like UV treatment, DMSO-induced telomerase activity peaked at 8 h following treatment (Fig. 1B). While DMSO treatment also appeared to be dose-related, the increases in telomerase activity ranged from 2.5-fold

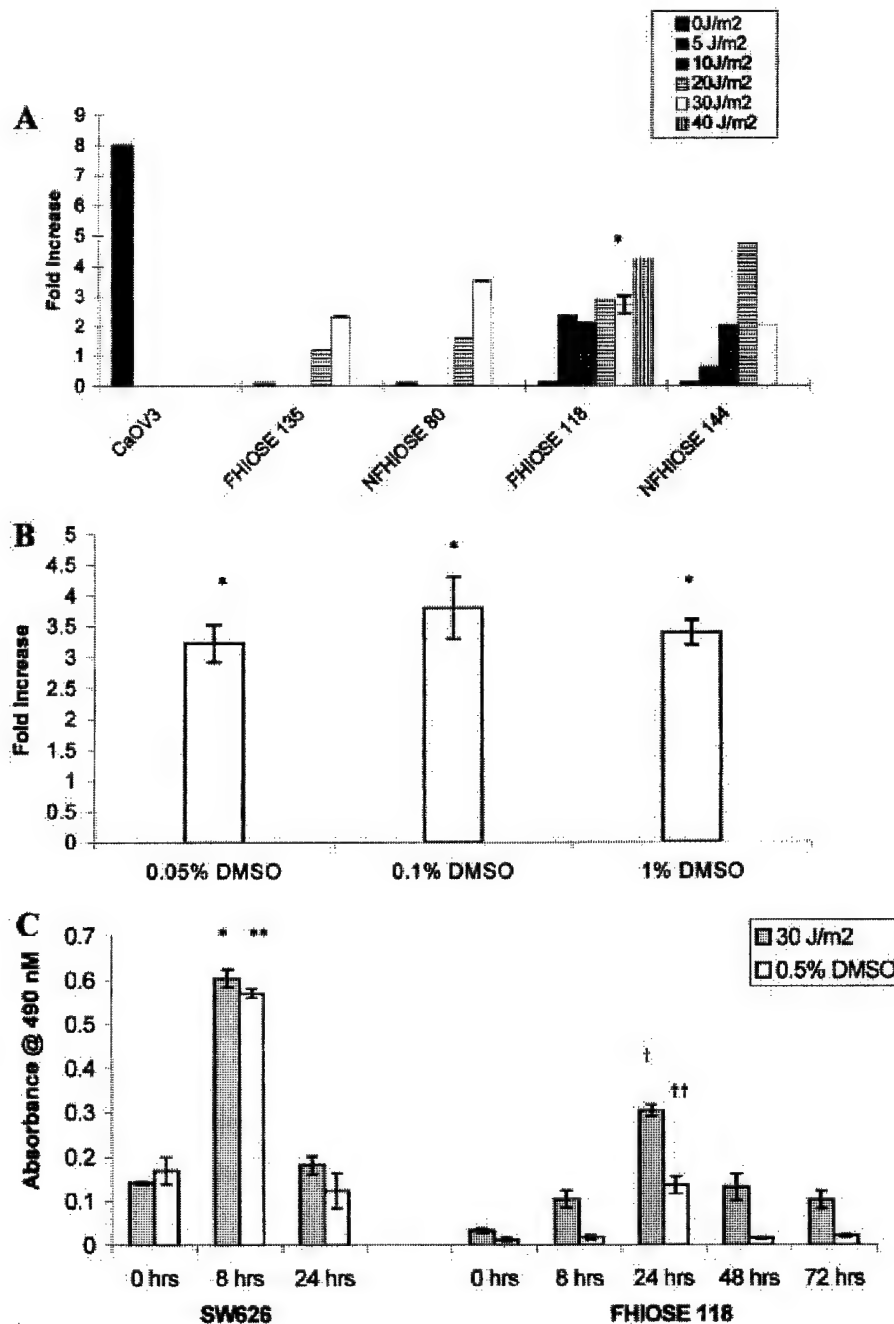


FIG. 2. UV- and DMSO-induced telomerase activity in the IOSE cell lines. Values were expressed as a fold increase above control levels, with the control value used as the denominator for the determination of fold increase of the treated samples. (A) UV-induced telomerase activity in IOSE cells was measured at 24 h following 0, 5, 10, 20, 30, and 40 J/m² UV from duplicate samples of IOSE cell lines, and the results are expressed as fold increase. SE was only determined in one instance. * $P \leq 0.02$. (B) DMSO-induced telomerase activity in FHIOSE 118 cells following 0.05, 0.1, and 1.0% DMSO. Triplicate samples were assayed for telomerase activity, and the results are expressed as fold increase \pm SE. * $P \leq 0.005$. (C) Comparison of UV- and DMSO-induced telomerase activity in SW626 cells at 0, 8, and 24 h and in FHIOSE 118 cells at 0, 8, 24, 48, and 72 h following treatment with 30 J/m² UV or 0.5% DMSO. * $P \leq 0.009$, ** $P \leq 0.001$, † $P \leq 0.02$, ‡ $P \leq 0.003$.

with 0.07% DMSO to 5-fold with 1% DMSO (Fig. 1B). Further, both UV- and DMSO-induced telomerase activity was transient in SW626 cells, since the elevated levels of telomerase activity returned to near basal levels within 24 h of UV or DMSO treatment (Figs. 1A and 2C).

We expanded our studies to determine whether UV or DMSO could activate telomerase activity in telomerase-negative, nontumorigenic ovarian epithelial cell lines. In contrast to the highly telomerase-positive CaOV3 ovarian cancer cells, which served as our internal control, the levels of telomerase detected for all four

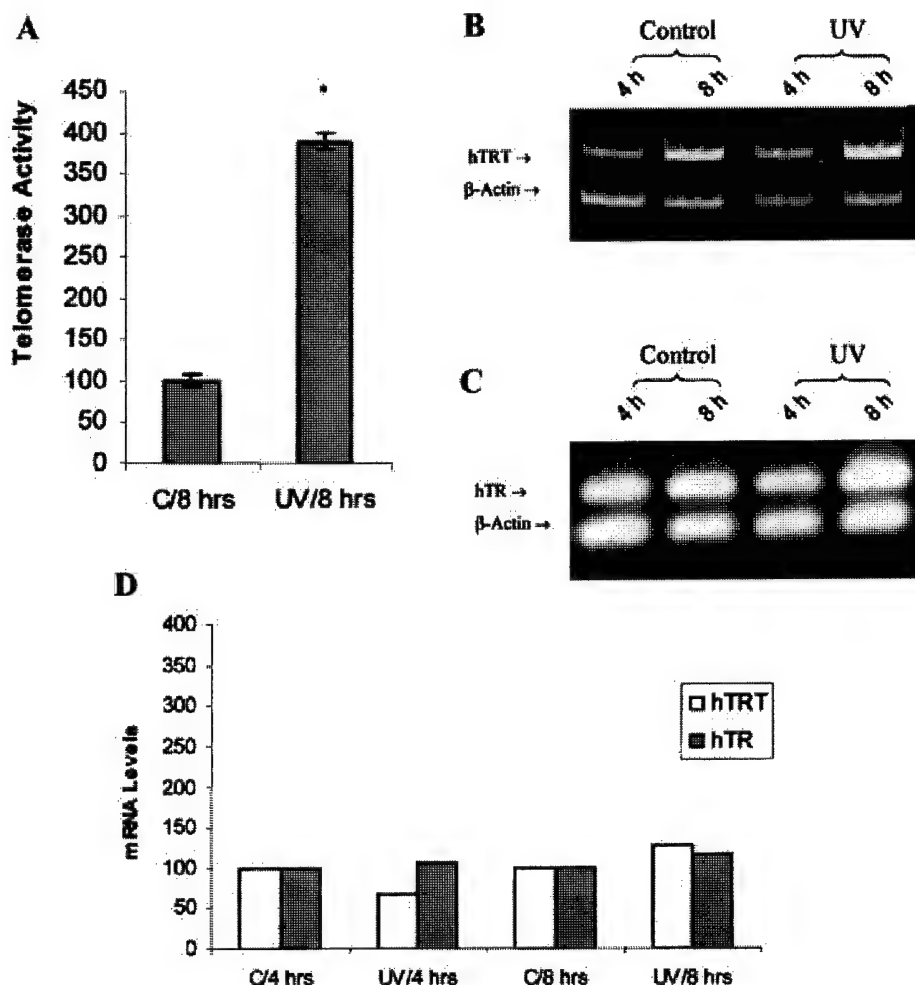


FIG. 3. hTERT and hTR mRNA levels following UV-induced telomerase activity in SW626 cells. (A) SW626 cells were treated with 30 J/m² UV and assayed for telomerase activity by PCR-ELISA 8 h following treatment. Samples were assayed in triplicate and expressed as mean percentage of control ± SE (C, control). **P* ≤ 0.005. (B) PAGE following RT-PCR revealed a 145-bp hTERT product and a 98-bp β-actin product. (C) PAGE following RT-PCR revealed a 103-bp hTR product and a 98-bp β-actin product. (D) Graphical representation of hTERT and hTR mRNA net intensities in treated and untreated samples relative to β-actin mRNAs.

untreated IOSE cell lines examined (FHIOSE 135, FHIOSE 118, NFHIOSE 80, and NFHIOSE 144) were similar to the assay background levels, defined by the assay as "telomerase-negative." Following UV or DMSO treatment, all IOSE cell lines exhibited detectable levels of telomerase activity (Figs. 2A and 2B). Although a dose-dependent relationship was not observed in IOSE cells, treatment with 5–40 J/m² UV induced a 1.5- to 5.5-fold increase in telomerase activity (Fig. 2A). Treatment with varying doses of DMSO resulted in a 2.7- to 2.9-fold induction of telomerase activity in a dose-independent manner (Fig. 2B).

While UV or DMSO induced telomerase activity in both SW626 and FHIOSE118 cell lines, the absolute amount of telomerase activity induced was dramatically different between the two cell lines. In order to demonstrate this difference in telomerase levels,

SW626 and FHIOSE 118 cell lines were treated with UV or DMSO and absorbance values of the assay were compared directly (Fig. 2C). Treatment with 0.5% DMSO or 30 J/m² UV resulted in a 3- to 4-fold increase over endogenous telomerase activity in SW626 cells by 8 h (Fig. 2C), returning to control levels by 24 h. In FHIOSE 118 cells, a 9- to 10-fold increase in telomerase activity was observed at 24 h following UV or 0.5% DMSO treatment (Fig. 2C). In contrast to SW626 cells, FHIOSE 118 cells demonstrated maximal induction of telomerase activity at 24 h following UV or DMSO treatment (Fig. 2C). Telomerase activity was lost in FHIOSE 118 cells within 48 h following treatment with DMSO. Forty-eight hours following UV treatment, the FHIOSE 118 cells continued to exhibit progressively decreasing telomerase levels. These data suggest that UV- and DMSO-induced telomerase activity may be a transient event in both cell lines.

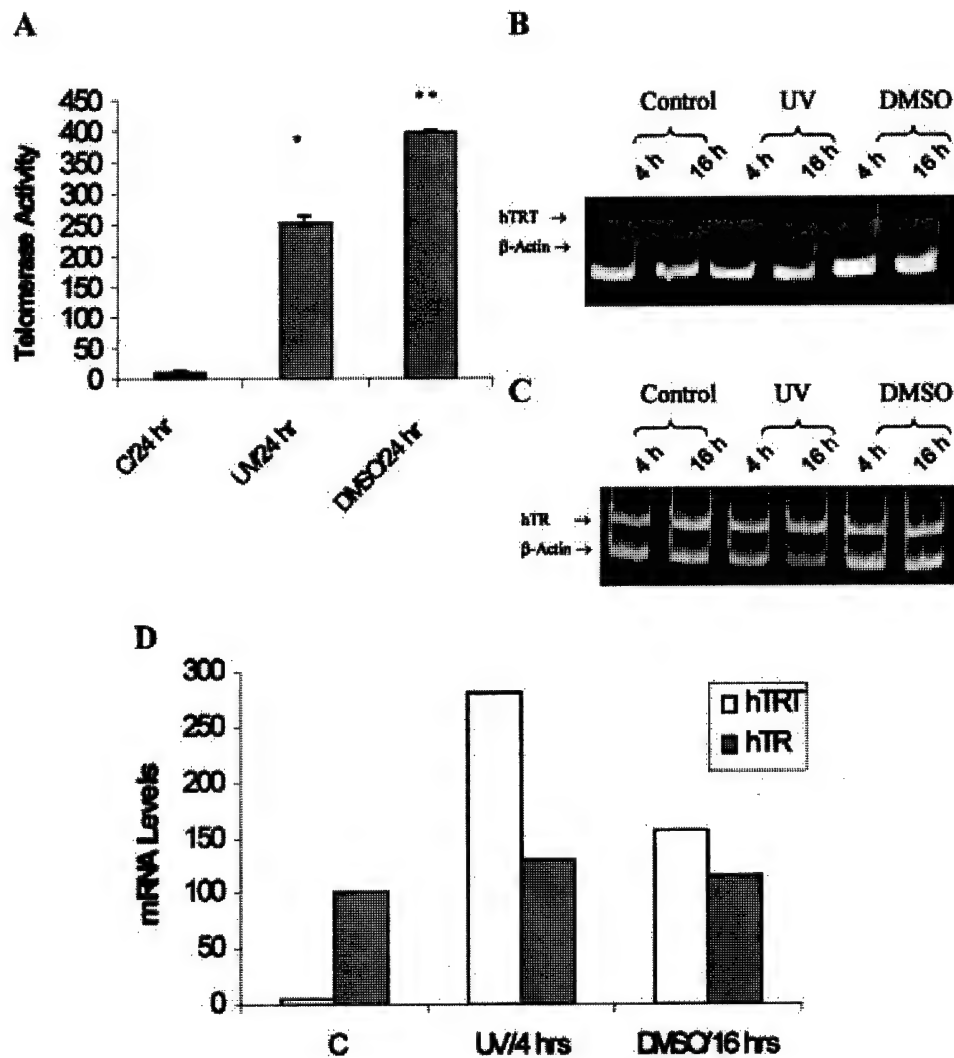


FIG. 4. hTERT and hTR mRNA levels following UV- and DMSO-induced telomerase activity in FHIOSE 118 cells. (A) FHIOSE 118 cells were treated with 30 J/m² UV or 0.5% DMSO and assayed for telomerase activity by PCR-ELISA 24 h following treatment. Telomerase activity was expressed as a fold increase above control (C) levels, with the control value used as the denominator for the determination of fold increase of the treated samples. Samples were assayed in triplicate and expressed as mean percentage of control \pm SE. * $P \leq 0.05$, ** $P \leq 0.005$ (B) PAGE following RT-PCR revealed a 145-bp hTERT product and a 98-bp β -actin product in UV and DMSO-treated samples at 4 and 16 h, respectively, but only the 98-bp β -actin product was detected in untreated FHIOSE 118 cells. (C) PAGE following RT-PCR revealed a 103-bp hTR product and a 98-bp β -actin product in all samples. (D) Graphical representation of hTERT and hTR net intensities in treated and untreated samples relative to β -actin mRNAs.

UV and DMSO induction of telomerase activity is transcription-dependent in FHIOSE 118 cells but not in SW626 cells. To examine the role of transcriptional control in UV- and DMSO-induced telomerase activity, RT-PCR was employed to determine the relative levels of the telomerase catalytic component (hTERT) and the RNA component (hTR) mRNA in SW626 and FHIOSE 118 cells (Figs. 3 and 4). SW626 cells were irradiated with 30 J/m² and assayed for telomerase activity at 8 h following treatment (Fig. 3A). A 3.75-fold increase in telomerase activity was detected between the control sample and the UV-irradiated sample. With telomerase levels peaking at 8 h in the SW626 cells, it was necessary to begin collection points prior to those times

in order to detect a change in mRNA levels. RT-PCR and PAGE analyses of parallel cultures revealed a 145- and a 98-bp band, representing the hTERT and β -actin products, respectively, at 4 and 8 h (Figs. 3B and 3C). However, despite the 3.75-fold increase in telomerase activity, no significant differences in the relative levels of hTERT transcript were observed between the control and UV-irradiated samples (Figs. 3B and 3D). RT-PCR and PAGE analyses also showed that there appeared to be no difference in the level of hTR transcript between the control and UV-irradiated SW626 samples (hTR transcript levels never exceeding 10% that of the controls) (Figs. 3C and 3D).

RT-PCR was similarly utilized to determine the rel-

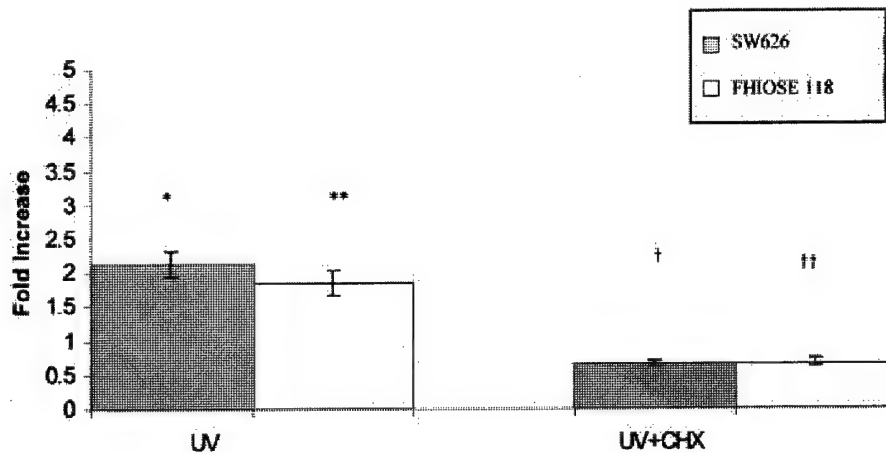


FIG. 5. Effect of cycloheximide on UV-induced telomerase activity. SW626 and FHIOSE 118 cells were treated with 40 μ M CHX, as described under Methods and Materials, treated with 30 J/m² UV, and subsequently assayed for telomerase activity. Absorbance values of the control samples were used as the denominator for the determination of fold increase of the treated samples. Results are triplicate samples expressed as fold increase \pm SE. * $P \leq 0.05$, ** $P \leq 0.02$, † $P \leq 0.03$, ‡ $P \leq 0.008$.

active levels of hTERT and hTR mRNAs in FHIOSE 118 cells following UV- and DMSO-induced *de novo* telomerase activity (Fig. 4). FHIOSE 118 cells were assayed for telomerase activity 24 h following treatment with either 30 J/m² UV or 0.5% DMSO. Both UV and DMSO treatment resulted in a significant induction of telomerase activity (Fig. 4A). UV caused a 2.5-fold induction of telomerase activity and treatment with DMSO resulted in a 4-fold induction compared to control levels. With telomerase levels requiring 24 h for maximal activity in the FHIOSE 118 cells, 4- and 16-h time points were selected in order to detect a change in mRNA levels. While the 98-bp band representing β -actin mRNA product was seen in all lanes, RT-PCR and PAGE analysis did not detect hTERT transcripts in untreated FHIOSE 118 cells (Figs. 4B and 4D). However, the 145-bp hTERT mRNA band was clearly visible in UV- and DMSO-treated samples collected at 4 and 16 h following treatment (Figs. 4B and 4D). Maximal hTERT mRNA levels were noted at 4 h following UV, having increased 280% above the control levels. hTERT mRNA levels steadily declined by 16 h following UV, decreasing to 156% (Fig. 4B) above the control levels. For DMSO-treated samples, peak hTERT transcript levels were observed at 16 h following treatment, having increased 300% above control levels. RT-PCR and PAGE analyses also revealed the constitutive expression of the 103- and 98-bp bands representing hTR and β -actin mRNA products, respectively, in treated and untreated FHIOSE 118 cells (Fig. 4C). Unlike hTERT mRNA levels, there was no apparent change in the levels of hTR transcripts between the control and UV-irradiated or DMSO-treated samples (Figs. 4C and 4D). Therefore, it appears that induced telomerase activity was preceded by an increase in hTERT transcript levels in FHIOSE 118 cells.

Induction of telomerase activity is partly dependent upon protein synthesis in SW626 and FHIOSE 118 cells. To determine whether protein synthesis was required for telomerase induction, the protein synthesis inhibitor CHX was used to inhibit *de novo* protein synthesis in SW626 and FHIOSE 118 cells following treatment with 30 J/m² UV (Fig. 5). In this set of experiments, UV-treated SW626 and FHIOSE 118 cells demonstrated an approximate twofold elevation in telomerase activity following treatment with 30 J/m² (Fig. 5). CHX inhibited UV-induced telomerase activity, decreasing SW626 telomerase levels to 50% of the control level by 8 h. However, CHX did not suppress basal telomerase activity in SW626 cells (data not shown). Treatment with CHX partially inhibited UV-induced telomerase activity in FHIOSE 118 cells but did not completely abolish UV-induced telomerase activity. Specifically, CHX suppressed UV-induced telomerase activity in FHIOSE 118 cells by 60%. For fold increase calculations, absorbance values of the control samples were used as the denominator for the determination of fold increase of the treated samples.

PI3K activity is increased following UV and DMSO treatment. Although UV is known to activate PI3K [27–33], it was necessary to determine whether DMSO was capable of activating PI3K. To compare the levels of PI3K activation between UV and DMSO, PI 3-kinase assays were performed following treatment of SW626 and FHIOSE 118 cells with UV or DMSO, respectively (Fig. 6). Since kinases are rapidly activated, samples were collected 30 min following treatment, when the kinases were maximally activated. Here, SW626 cells were treated with 30 J/m² UV and collected at 30 min following treatment. Similarly, FHIOSE 118 cells were treated with DMSO and collected 30 min following

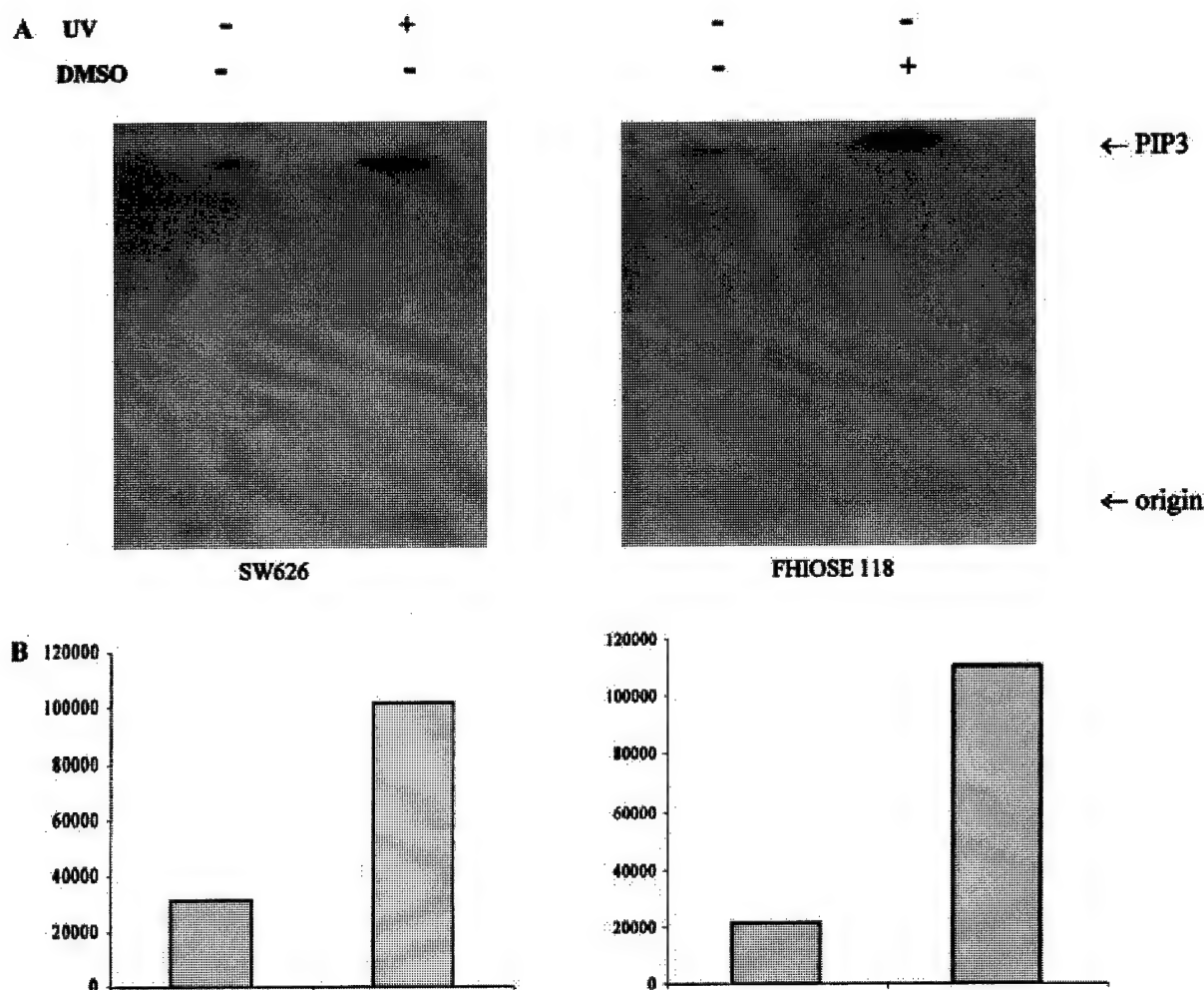


FIG. 6. PI 3-kinase activation precedes UV- and DMSO-induced telomerase activity. (A) SW626 cells were treated with 30 J/m² UV and FHIOSE 118 cells were treated with 0.05% DMSO. Cells were collected and assayed for PI3K activity as described under Methods and Materials. The conversion of PI(4,5)P₂ to PI 3-phosphate was determined by autoradiography. (B) ImageQuant software was used to quantify the PI 3-phosphate product. Graphical representation depicts band intensities as measured by the imaging software.

treatment. Lysates were then assayed for PI3K activity as previously described. In SW626 cells, PI3K activity increased 3-fold following UV treatment. Following treatment with DMSO, FHIOSE 118 cells demonstrated a 5-fold increased in PI3K activity, demonstrating an increase in PI3K activity above that seen with 30 J/m² UV. To the best of our knowledge, this is the first demonstration of DMSO-induced PI3K activity.

Inhibition of PI3K suppresses hTERT transcription in FHIOSE 118 cells but not in SW626 cells. In order to confirm the involvement of the PI3K signaling pathway for telomerase regulation in our model system, the PI3K inhibitors WM and LY were used. Using absorbance values of the control samples as the denominator for the determination of fold increase of the treated samples, a 2-fold elevation in telomerase activity was observed following treatment with 30 J/m² UV or 0.5% DMSO in SW626 cells (Fig. 7A). However, LY and WM suppressed UV- and DMSO-induced telomerase activ-

ity to below basal levels. The SW626 cells treated only with PI3K inhibitors demonstrated a 40% reduction in the basal telomerase activity. FHIOSE 118 cells treated with 30 J/m² UV or 0.5% DMSO demonstrated 3.3- and a 2-fold inductions, respectively, in telomerase activity above background (Fig. 7B). As with SW626 cells, LY blocked UV- and DMSO-induced telomerase activity in FHIOSE 118 cells by 250 ($P \leq 0.01$) and 175% ($P \leq 0.05$), respectively. It should be noted that treatment with either WM or LY did not result in increased cell death, as determined by trypan blue exclusion (data not shown).

To determine if PI3K inhibitors abrogated telomerase induction by affecting the transcription of telomerase components, RT-PCR for hTERT and hTR mRNAs was employed following treatment with LY in SW626 (Fig. 8) and FHIOSE 118 (Fig. 9) cells. Treatment with 10 μ M LY suppressed 60% of the basal telomerase activity in SW626 cells (Fig. 8A). However, LY-medi-

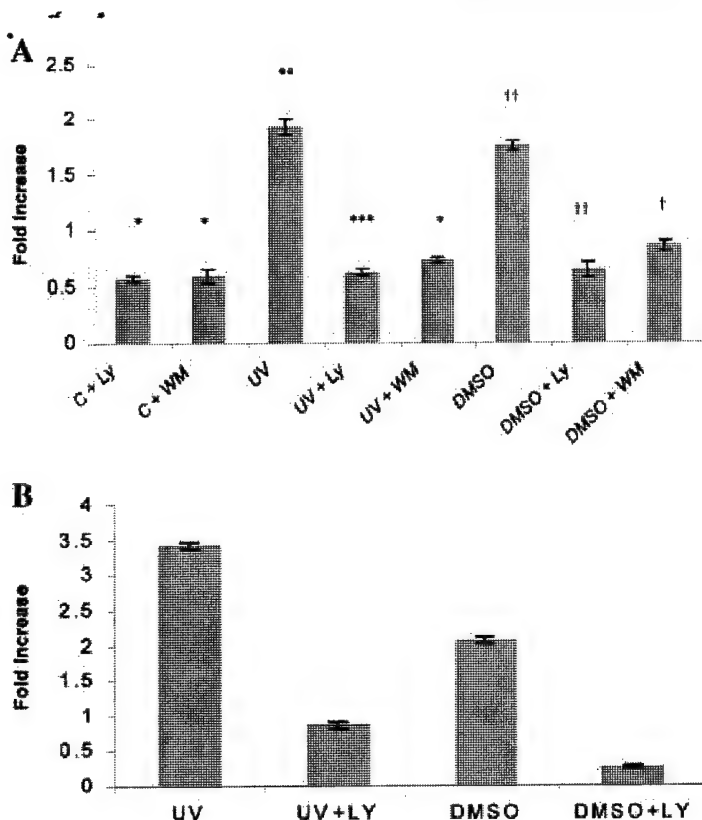


FIG. 7. Inhibition of PI3K inhibits UV or DMSO induction of telomerase activity. Telomerase activity is expressed as fold increase using the absorbance value of the control samples as the denominator for the determination of fold increase of the treated samples. (A) SW626 cells were treated with either 10 μ M LY or 100 nM WM, as described under Methods and Materials, exposed to either 30 J/m² UV or 0.5% DMSO, and subsequently assayed for telomerase activity. Results are triplicate samples expressed as fold increase \pm SE. * $P \leq 0.02$, ** $P \leq 0.002$, *** $P \leq 0.004$, † $P \leq 0.001$, †† $P \leq 0.003$ (B) FHI108 cells were treated with 10 μ M LY, treated with either 30 J/m² UV irradiation or 0.5% DMSO, and subsequently assayed for telomerase activity. $P \leq 0.05$ for UV + LY and DMSO + LY compared to their respective controls. Results are triplicate samples expressed as fold increase \pm SE.

ated suppression of telomerase activity in SW626 cells was not associated with any notable change in either hTERT or hTR mRNA levels, as determined by RT-PCR and PAGE analyses (Figs. 8B–8D).

Similarly, FHI108 cells were treated with UV in order to induce telomerase activity. UV treatment resulted in a 3.4-fold induction of telomerase ($P \leq 0.02$). Parallel cultures were treated with 10 μ M LY in addition to UV. Ten micromolar LY suppressed UV-induced telomerase activity approximately 250% in FHI108 cells ($P \leq 0.01$) (Fig. 9A), and this inhibition of telomerase induction was associated with decreased levels of hTERT mRNA (Figs. 9B–9D). This confirms a role for PI3K in telomerase regulation.

Chemotherapeutic agents do not induce telomerase activity. To determine if DNA damage could result in the induction of telomerase activity, SW626 cells were

treated with sublethal doses of a variety of chemotherapeutic agents and then assayed, in duplicate, for telomerase activity (Fig. 10). Sublethal doses of all chemotherapeutic agents were determined by performing a cell proliferation assay for each agent at the various concentrations used (data not shown). Changes in telomerase activity observed following treatment with 25 or 100 μ M CP, 10, 50, or 100 μ M ET, and 1, 10, or 25 μ M CB (Fig. 10) were not statistically significant ($P > 0.05$). Thus, assays were not performed in triplicate, and the standard errors are not shown.

DISCUSSION

Not only is telomerase expressed in over 90% of ovarian cancers, telomerase activity has also been shown to correlate well with ovarian tumor aggressiveness or stage [4, 25, 37–40]. Although information about telomerase regulation is incomplete, various studies propose a complex model of regulation [1, 10, 12, 13]. Given the important clinical connection between telomerase and ovarian cancer, it is imperative to devise a model system in which telomerase activity can be manipulated and studied. UV and DMSO were deemed to be potential modulators of telomerase activity in ovarian epithelial cell lines based upon our current understanding of telomerase regulation and ovarian neoplastic progression. Specifically, since Ueda *et al.* reported increased telomerase activity following UV exposure of normal skin [53] and Hande *et al.* reported a dose-dependent increase in telomerase activity following UV treatment in CHO cells [54], we sought to determine whether UV could induce telomerase activity in ovarian epithelial cell lines. Further, in contrast to carcinomas of most other tissues that de-differentiate with neoplastic progression, ovarian cancer progression is associated with the acquisition of morphological features characteristic of the endocervix, endometrium, and oviduct owing to their common embryonic origin [55–57]. For example, features associated with a more differentiated epithelial phenotype in ovarian neoplasia include expression of E-cadherin, activin receptors, $\alpha_v\beta_3$ -integrin, vitronectin, and CA125, products normally associated with the oviduct, endometrium, and endocervix [56–60]. Bearing this in mind, we also attempted to determine whether DMSO, a commonly employed differentiation-inducing agent, could induce telomerase activity by mimicking the neoplastic progression of ovarian surface epithelial cells. While we examined our cells for markers of differentiation such as E-cadherin expression, no morphological changes were detected (data not shown). However, DMSO was capable of inducing PI 3-kinase activity as well as telomerase activity. In contrast to our findings, others have reported telomerase suppression associated with DMSO-induced cellular differentiation

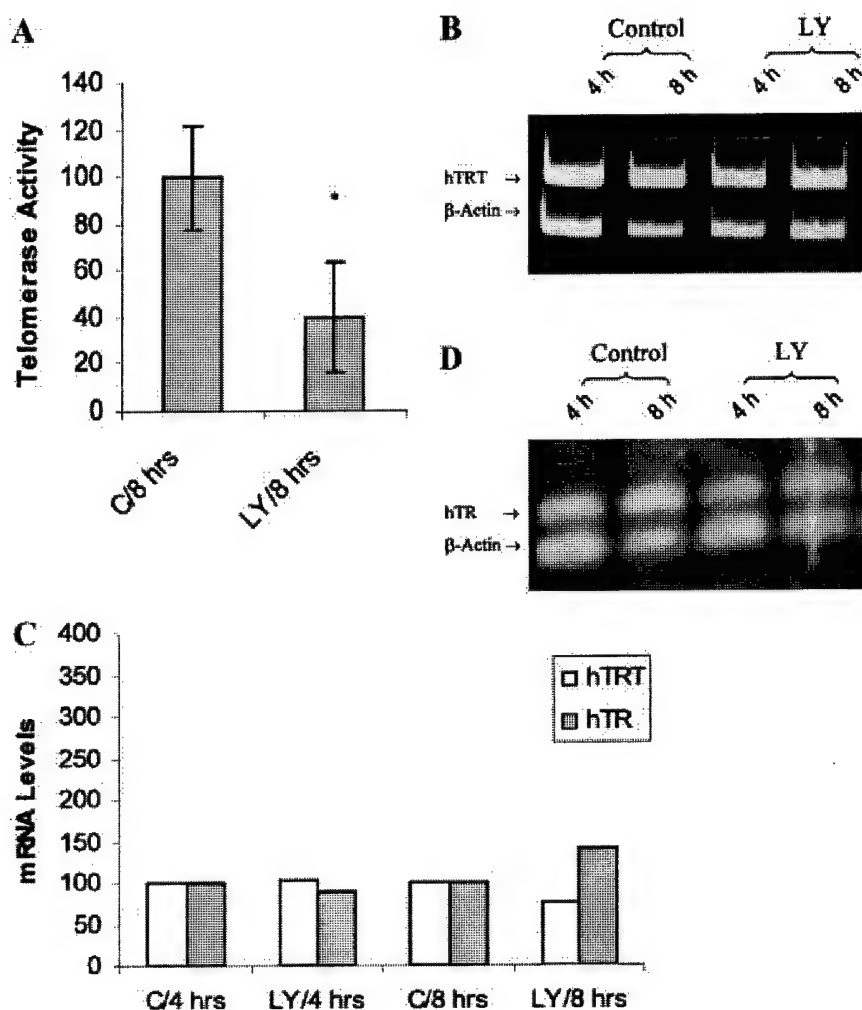


FIG. 8. hTERT and hTR mRNA levels in SW626 cells following LY-mediated suppression of telomerase activity. (A) SW626 cells were treated with 10 μ M LY and assayed for telomerase activity by PCR-ELISA at 8 h. Telomerase activity is expressed as fold increase using the absorbance values of the control (C) samples as the denominator for the determination of fold increase of the treated samples. Samples were assayed in triplicate and expressed as mean percentage of control \pm SE. * $P \leq 0.005$. (B) PAGE following RT-PCR revealed a 145-bp hTERT product and a 98-bp β -actin product in all samples. β -Actin was used as an internal control. (C) PAGE following RT-PCR revealed a 103-bp hTR product and a 98-bp β -actin product in all samples. β -Actin was used as an internal control. (D) Graphical representation of hTERT or hTR net intensity relative to β -actin mRNAs.

and/or G_0/G_1 cell cycle arrest in a variety of leukemia, colon carcinoma, teratocarcinoma, and lymphoma cells [15, 16]. Discrepancies in the effects of DMSO on telomerase activity observed by these authors and those in the present study may be due to variation in treatment methodologies. While other studies employ high-dose DMSO treatment for periods of days, our treatment involved much lower doses for a 2-h treatment period.

We have developed a culture model system in which we have shown, for the first time, that UV or DMSO can transiently induce *de novo* telomerase activity in previously telomerase-negative human ovarian cell lines as well as elevate endogenous telomerase activity in ovarian cancer cells. Using the very sensitive telomerase ELISA assay, changes in telomerase activity

were represented graphically as fold increase, where the absorbance values of the control samples were used as the denominator for the determination of fold increase of the treated samples. We found a dose-related elevation or induction of telomerase activity, following UV or DMSO treatment, in telomerase-positive SW626 and telomerase-negative FHIOSE 118 ovarian cell lines, respectively. UV-induced telomerase activity appeared to be greater in magnitude in FHIOSE 118 cells than in the SW626 cell line and may reflect the fact that FHIOSE cells are normally telomerase negative. In addition, we also found differences in the kinetics of UV- or DMSO-induced telomerase activity between telomerase-positive and -negative cell lines. SW626 cancer cells exhibited maximally elevated telomerase levels at 8 h following UV or DMSO, while maximal

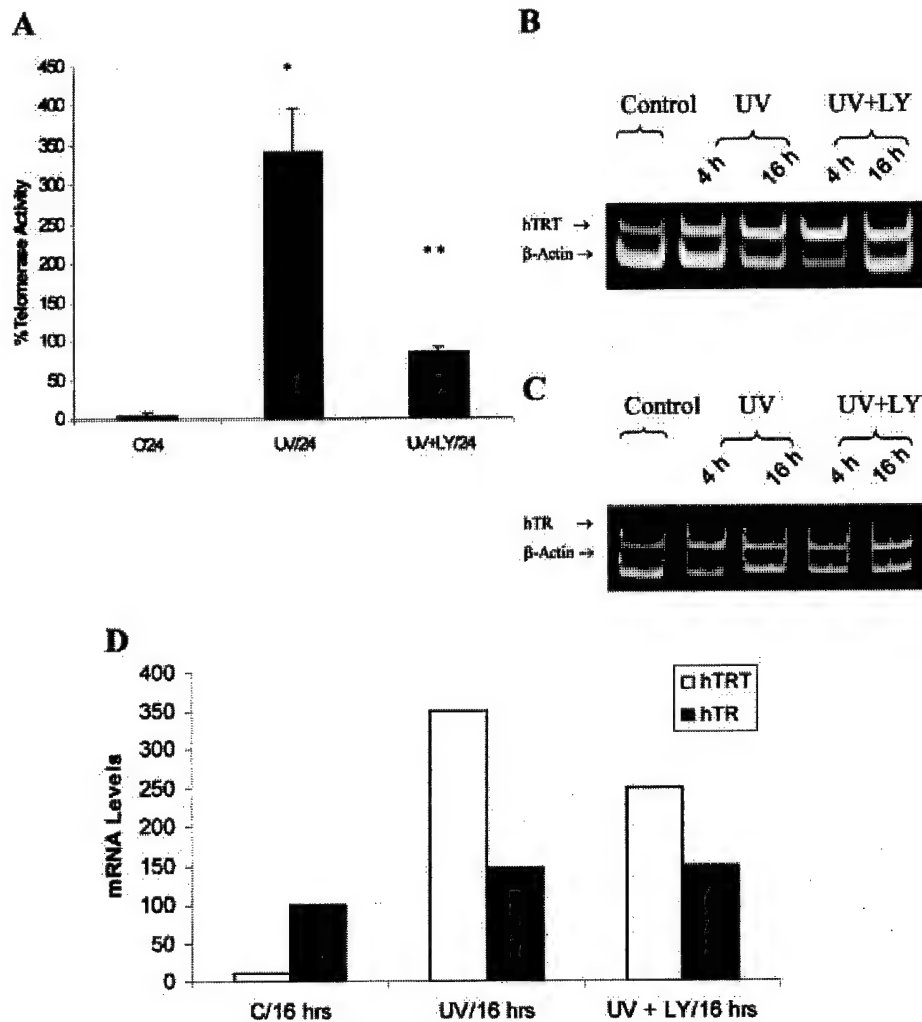


FIG. 9. hTERT and hTR mRNA levels in FHIOSE 118 cells following LY-mediated suppression of UV-induced telomerase activity. (A) FHIOSE 118 cells were treated with 30 J/m² UV. Telomerase activity is expressed as fold increase using the absorbance values of the control (C) samples as the denominator for the determination of fold increase of the treated samples. Triplicate samples were assayed for telomerase activity by PCR-ELISA and expressed as mean percent of control \pm SE. * $P \leq 0.02$, ** $P \leq 0.01$ (B) PAGE following RT-PCR revealed a 145-bp hTERT product in the treated samples. RT-PCR also revealed a 98-bp β -actin product in all samples. β -Actin was used as an internal control. (C) PAGE following RT-PCR revealed a 103-bp hTR product and a 98-bp β -actin product in all samples. β -Actin was used as an internal control. (D) Graphical representation of hTERT or hTR net intensity relative to β -actin mRNAs.

telomerase induction in FHIOSE 118 cells occurred only at 24 h following treatment. Furthermore, we found that UV- or DMSO-induced telomerase activity was transient in all cell lines examined. Induced telomerase activity progressively decreased in FHIOSE cells by 48 h after UV or DMSO treatment, while induced telomerase activity in SW626 cells returned to controls levels by 24 h after treatment. These results are in agreement with previous observations reporting a strong association between telomerase activity with sun exposure [53], a UV dose-dependent induction of telomerase activity in CHO cells [54], and restoration of basal telomerase levels within 24 h following withdrawal of DMSO [61].

We used our model system to identify some of the components that may regulate telomerase activity. We

found that SW626 cells constitutively expressed both hTERT and hTR mRNA. DMSO- or UV-induced elevation of telomerase was not associated with an increase in either hTERT or hTR transcription, suggesting that SW626 may constitutively express maximal levels of hTERT and hTR mRNAs. In contrast, UV- or DMSO-induced *de novo* telomerase activity in FHIOSE 118 cells required transcription of hTERT, but not hTR, mRNA. This finding may reflect the lag time for telomerase induction in these cells. Again, our results are in agreement with those of others, which suggest that while cells often constitutively express the hTR component of telomerase, hTERT expression is the rate-limiting factor in telomerase activity [1, 3–5, 51, 62].

Furthermore, we also showed that protein synthesis was required for *de novo* telomerase activity in

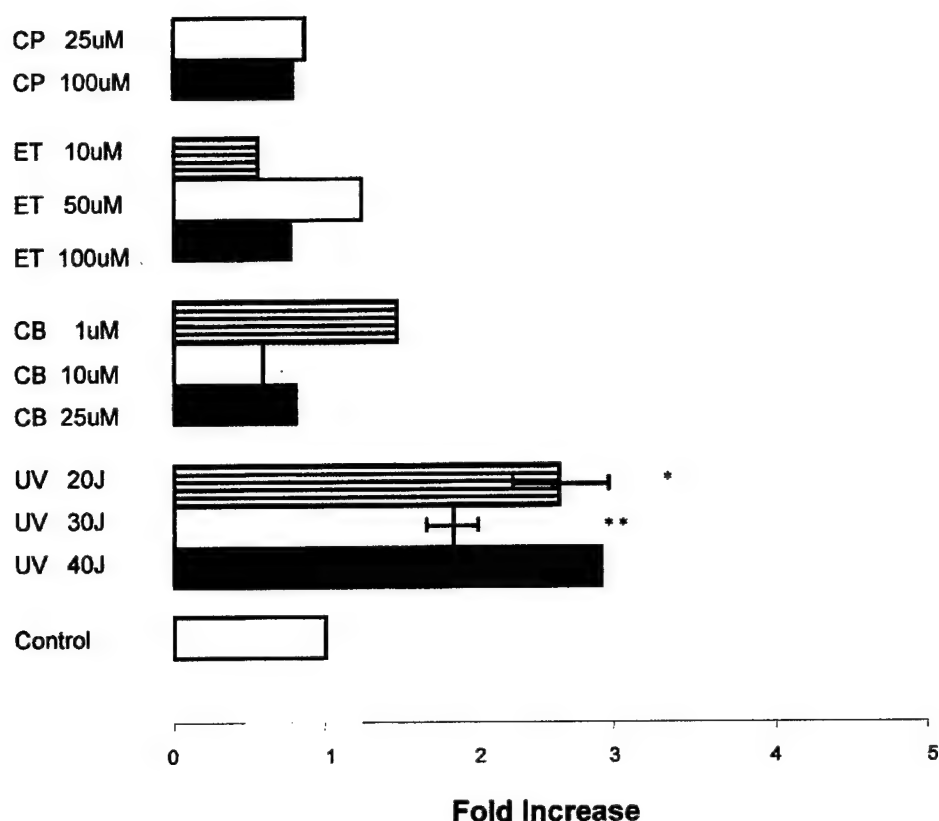


FIG. 10. Effect of chemotherapeutic agents on telomerase activity in SW626 cells. Cells were treated as described under Methods and Materials. Telomerase activity is expressed as fold increase using the absorbance values of the control samples as the denominator for the determination of fold increase of the treated samples. Only 20- and 30-J/m² samples were assayed in triplicate, the remainder were in duplicate. Fold increases in telomerase activity are shown \pm SE. * $P \leq 0.04$, ** $P \leq 0.03$.

FHIOSE 118 cells as well as for elevating endogenous telomerase activity in SW626 cells. CHX inhibited UV-induced telomerase activity in FHIOSE 118 cells by 60% and diminished telomerase activity in SW626 cells to 50% of control levels. The inability of CHX to completely abolish telomerase activity may be due to the 24-h half-life of telomerase [63]. While the exact role of protein synthesis in telomerase regulation remains unclear, CHX is a general protein synthesis inhibitor and its inhibitory action may prevent synthesis of the telomerase catalytic component, hTERT, and/or associated proteins such as tankyrase, TLP1, or as of yet unidentified components of the telomerase complex. Therefore, cells without prior detectable telomerase activity, such as FHIOSE 118 cells, would be required to assemble telomerase from *de novo* transcription, translation, and assembly of its component parts.

PI3K can be activated by exogenously applied cellular stress [64] and it has been implicated in telomerase regulation [20]. Akt, a down-stream target of PI3K, has also been implicated in the activation of telomerase by direct phosphorylation of the hTERT catalytic component [24]. We also examined the relative contribution of PI3K to telomerase regulation in our model system. PI 3-kinase assays revealed that PI3K levels were in-

creased following UV exposure, as previously reported [27–33]. In addition, we report here, for the first time, that DMSO can increase PI3K activity levels. Using WM or LY, both specific inhibitors of PI3K, we were able to demonstrate inhibition of UV- or DMSO-induced telomerase activity in both SW626 and FHIOSE 118 cells. This inhibition of telomerase activity through inhibition of PI3K occurred independent of changes in hTERT and hTR transcription levels. These results suggest that, perhaps, at least initiation of telomerase activity in FHIOSE 118 cells may involve the same signal transduction pathway that regulates cancer cells. These findings also suggest that there may be an additional component in telomerase regulation, such as posttranslational modification by phosphorylation of one or more of the telomerase components.

While the exact mechanism(s) of telomerase activation is not completely understood, the TTAGGG telomeric repeat sequence has been shown to be a hot spot for chromosomal breakage [65, 66], a target for UV-induced pyrimidine dimers, as well as a target for CP- or CB-induced intrastrand d(GpG) and d(ApG) cross-links [67, 68]. Consequently, commonly used chemotherapeutic agents might increase telomerase activity and potentially undermine chemotherapeutic efficacy.

Therefore, we also sought to determine whether chemotherapeutic agents would induce telomerase activity in our model system. We treated SW626 cells for a period of time previously found to be sufficient to induce intrastrand cross-links without causing significant cellular lethality [68]. We were unable to suppress endogenous telomerase activity in SW626 cancer cells with comparable doses of chemotherapeutic agents, as has been reported elsewhere [69]. This may be attributed to inherent differences in the cell types used as well as the length of exposure to the drug employed. Taken together, our results suggest that enhanced telomerase activity following cellular perturbation is specific to certain exogenous applied agents. Further, our results support current therapeutic modalities for ovarian cancer, since use of these chemotherapeutic agents does not appear to promote telomerase activity, decreasing the probability of telomerase-promoted tumor cell survival.

In summary, we have developed a novel and useful culture model system, for elevating telomerase activity in a telomerase-positive cell line, and, most importantly, for demonstrating *de novo* telomerase activity in a telomerase-negative cell line using UV or DMSO. The exact mechanism of telomerase regulation in our model system is not completely known, and clearly further studies are warranted. It appears that UV and DMSO induction of telomerase activity begins a signaling cascade, originating at the cellular membrane, via membrane perturbation caused by UV or DMSO [70–74]. This, in turn, may activate telomerase activity through PI3K, possibly by phosphorylation of the hTERT component. While maintenance of telomerase levels may require only protein synthesis and post-translational modifications such as phosphorylation, initiation of *de novo* telomerase activity requires hTERT transcription and protein synthesis. Lastly, the lack of telomerase induction by chemotherapeutic agents in our model system will allow for additional studies aimed at determining clinically feasible mechanisms to suppress telomerase in cancer cells without reducing the efficacy of conventional chemotherapy.

We thank Dr. Santo Nicosia and Mrs. Nancy Lowell for comments on the manuscript and Mr. Zheng Qiang Yuan for assistance with the PI3K assays. Cycloheximide was generously provided by Dr. Ken Keller (USF). This work was supported by a New Investigator Award from the H. Lee Moffitt Cancer Center and Grant No. F99USF-2 from the American Cancer Society, Florida Division, to P.A.K.

REFERENCES

1. Ulaner, G. A., Hu, J. F., Vu, T. H., Oruganti, H., Giudice, L. C., and Hoffman, A. R. (2000). Regulation of telomerase by alternate splicing of human telomerase reverse transcriptase (hTERT) in normal and neoplastic ovary, endometrium and myometrium. *Int. J. Cancer* **85**, 330–335.
2. Ito, H., Kyo, S., Kanaya, T., Takakura, M., Inoue, M., and Namiki, M. (1998). Expression of human telomerase subunits and correlation with telomerase activity in urothelial cancer. *Clin. Cancer Res.* **4**: 1603–1608.
3. Kanaya, T., Kyo, S., Takakura, M., Ito, H., Namiki, M., and Inoue, M. (1998). hTERT is a critical determinant of telomerase activity in renal cell carcinoma. *Int. J. Cancer* **79**: 539–543.
4. Kakakura, M., Kyo, S., Kanaya, T., Tanaka, M., and Inoue, M. (1998). Expression of human telomerase subunits and correlation with telomerase activity in cervical cancer. *Cancer Res.* **58**: 1558–1561.
5. Weinrich, S. L., Pruzan, R., Ma, L., Ouellette, M., Tesmer, V. M., Holt, S. E., Bodnar, A. G., Lichtsteiner, S., Kim, N. W., Trager, J. B., Taylor, R. D., Carlos, R., Andrews, W. H., Wright, W. E., Shay, J. W., Harley, C. B., and Morin, G. B. (1997). Reconstitution of human telomerase with template RNA component hTR and the catalytic protein subunit hTERT. *Nat. Genet.* **17**: 498–502.
6. Smogorzewska, A., van Steensel, B., Bianchi, A., Oelmann, S., Schaefer, M. R., Schnapp, G., and de Lange, T. (2000). Control of human telomerase length by TRF1 and TRF2. *Mol. Cell. Biol.* **20**: 1659–1658.
7. Bianchi, A., Stansel, R. M., Fairall, L., Griffith, J. D., Rhodes, D., and de Lange, T. (1999). TRF1 binds a bipartite telomeric site with extreme spatial flexibility. *EMBO J.* **18**: 5735–5744.
8. Zhu, X. D., Kuster, B., Mann, M., Petrini, J. H., and de Lange, T. (2000). Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. *Nat. Genet.* **25**: 347–352.
9. Smith, S., Giriat, L., Schmitt, A., and de Lange, T. (1998). Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science* **282**: 1484–1487.
10. Buchkovich, K. J., and Greider, C. W. (1996). Telomerase regulation during entry into the cell cycle in normal human T cells. *Mol. Biol. Cell* **7**: 1443–1454.
11. Terasawa, K., Sagae, S., Takeda, T., Ishioka, S., Kobayashi, K., and Kudo, R. (1999). Telomerase activity in malignant ovarian tumors with deregulation of cell cycle regulatory proteins. *Cancer Lett.* **142**: 207–217.
12. Holt, S. E., Aisner, D. L., Shay, J. W., and Wright, W. E. (1997). Lack of cell cycle regulation of telomerase activity in human cells. *Proc. Natl. Acad. Sci. USA* **94**: 10687–10692.
13. Holt, S. E., Wright, W. E., and Shay, J. W. (1996). Regulation of telomerase activity in immortal cell lines. *Mol. Cell. Biol.* **16**: 2932–2939.
14. Letaurtre, F., Gluckman, E., Li, X., and Carosella, E. (1997). Telomerase activity during the cell cycle and in gamma-irradiated hematopoietic cells. *Leukemia* **11**: 1681–1689.
15. Bestilny, L. J., Brown, C. B., Miura, Y., and Robertson, L. D. (1996). Selective inhibition of telomerase activity during terminal differentiation of immortal cell lines. *Cancer Res.* **56**: 3796–3802.
16. Sharma, H. W., Sokoloski, H. A., Perez, J. R., Maltese, J. Y., Sartorelli, A. C., Stein, C. A., Nichols, G., Khaled, Z., Telang, N. T., and Narayanan, R. (1995). Differentiation of immortal cells inhibits telomerase activity. *Proc. Natl. Acad. Sci. USA* **92**: 12343–12346.
17. Pai, R. B., Pai, S., Kukhanova, M., Dutschman, G. E., Guo, X., and Cheng, Y. C. (1998). Telomerase from human leukemia cells: Properties and its interaction with deoxynucleoside analogues. *Cancer Res.* **58**: 1909–1913.
18. Naasani, I., Seimiya, H., and Tsuruo, T. (1998). Telomerase inhibition, telomere shortening, and senescence of cancer cells

- by tea catechins. *Biochem. Biophys. Res. Commun.* **249**: 391–396.
19. Gomez, D. E., Tehera, A. M., and Olivero, O. A. (1998). Irreversible telomere shortening by 3'-azido-2', 3'-dideoxythymidine treatment. *Biochem. Biophys. Res. Commun.* **246**: 107–110.
20. Devereux, T. R., Horikawa, I., Anna, C. H., Annab, L. A., Afshari, C. A., and Barrett, J. C. (1999). DNA methylation of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. *Cancer Res.* **59**: 6087–6090.
21. Oh, S., Song, Y. H., Yim, J., and Kim, T. K. (1999). In vivo and in vitro analysis of Myc for differential promoter activities of the human telomerase (hTERT) gene in normal and tumor cells. *Biochem. Biophys. Res. Commun.* **263**: 361–365.
22. Wu, K. J., Grandori, C., Amacker, M., Simon, V. N., Polack, A., Linger, J., and Dalla-Favera, R. (1999). Direct activation of TERT transcription by c-MYC. *Nat. Genet.* **21**: 220–224.
23. Greenberg, R. A., O'Hagan, R. C., Deng, H., Xiao, Q., Hann, S. R., Adams, R. R., Lichtsteiner, S., Chin, L., Morin, G. B., and DePinho, R. A. (1999). Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. *Oncogene* **18**: 1219–1226.
24. Kang, S. S., Kwon, T., Kwon, D. Y., and Do, S. (1999). Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J. Biol. Chem.* **274**: 13085–13090.
25. Shay, J. W., and Bacchetti, S. (1997). A survey of telomerase activity in human cancer. *Eur. J. Cancer* **33**: 787–791.
26. Cheng, J. Q., Altomare, D. A., Klein, M. A., Lee, W. C., Kruh, G. D., Lissy, N. A., and Testa, J. R. (1997). Transforming activity and mitosis-related expression of the AKT2 oncogene: Evidence suggesting a link between cell cycle regulation and oncogenesis. *Oncogene* **14**: 2793–2801.
27. Tachiiri, S., Sasai, K., Oya, N., and Hiraoka, M. (2000). Enhanced cell killing by overexpression of dominant-negative phosphatidylinositol 3-kinase subunit, Deltap85, following genotoxic stresses. *Jpn. J. Cancer Res.* **12**: 1314–1318.
28. Okishio, N., Nagai, M., Fukuda, R., Nagatomo, S., and Kitagawa, T. (2000). Interactions of phosphatidylinositol 3-kinase Src homology 3 domain with its ligand peptide studied by absorption, circular dichroism, and UV resonance raman spectroscopies. *Biopolymers* **57**: 208–217.
29. Kabuyama, Y., Hamaya, M., and Homa, Y. (1998). Wavelength specific activation of PI 3-kinase by UVB irradiation. *FEBS Lett.* **441**: 297–301.
30. Wan, Y. S., Wang, Z. Q., Shao, Y., Voorhees, J. J., and Fisher, G. J. (2001). Ultraviolet irradiation activates PI 3-kinase/AKT survival pathway via EGF receptors in human skin in vivo. *Int. J. Oncol.* **3**: 461–466.
31. Nghiem, P. (2000). Identifying the missing link between UV and p53: The role of phosphatidylinositol kinase-related kinases. *J. Am. Acad. Dermatol.* **43**: 706–707.
32. Piacentini, M. P., Ricci, D., Fraternale, D., Piatti, E., Manunta, A., and Accorsi, A. (1999). Effects of UV-C irradiation on phosphoinositide turnover in plant cells: Similarities with those occurring via the formation of reactive oxygen intermediates in animal cells. *Comp. Biochem. Physiol. Biochem. Mol. Biol.* **122**: 293–299.
33. Jbuki, Y., and Goto, R. (2000). Suppression of apoptosis by UVB irradiation: Survival signaling via PI 3-kinase/AKT pathway. *Biochem. Biophys. Res. Commun.* **279**: 872–878.
34. Furlong, M. T., Hough, C. D., and Sherman-Baust, C. A. (1999). Evidence for the colonic origin of ovarian cancer cell line SW626. *J. Natl. Cancer Inst.* **91**: 1327–1328.
35. Maines-Bandiera, S. L., Kruk, P. A., and Auersperg, N. (1992). Simian virus 40-transformed human ovarian surface epithelial cells escape normal growth controls but retain morphogenetic responses to extracellular matrix. *Am. J. Obstet. Gynecol.* **167**: 729–735.
36. Kruk, P. A., Godwin, A. K., Hamilton, T. C., and Auersperg, N. (1999). Telomeric instability and reduced proliferative potential in ovarian surface epithelial cells from women with a family history of ovarian cancer. *Gynecol. Oncol.* **73**: 229–236.
37. Bechter, O. E., Eisterer, W., Pall, G., Hilbe, W., Kuehr, T., and Thaler, J. (1998). Telomere length and telomerase activity predict survival in patients with B cell chronic lymphocytic leukemia. *Cancer Res.* **58**: 4918–4922.
38. Bosserhoff, A. K., Giabia, A., and Stolz, W. (1997). Detection of telomerase activity in skin, melanocytic nevi, and melanoma by telomerase PCR ELISA. *Biochemica* **3**: 16–18.
39. Hoos, A., Hepp, H. H., Kaul, S., Ahlert, T., Bastert, G., and Wallwiener, D. (1998). Telomerase activity correlates with tumor aggressiveness and reflects therapy effect in breast cancer. *Int. J. Cancer* **79**: 8–12.
40. Muller, M., Heine, B., Heicappel, R., Emrich, T., Hummel, M., Stein, H., and Miller, K. (1996). Telomerase activity in bladder cancer, bladder washings and in urine. *Int. J. Oncol.* **9**: 1169–1173.
41. Sawant, S. G., Antonacci, R., and Pandita, T. K. (1997). Determination of telomerase activity in HeLa cells after treatment with ionizing radiation by telomerase PCR ELISA. *Biochemica* **4**: 22–24.
42. Colitz, C. M. H., Davidson, M. G., and McGahan, M. C. (1999). Telomerase activity in lens epithelial cells of normal and cataractous lenses. *Exp. Eye Res.* **69**: 641–649.
43. Obyashiki, J. H., Obyashiki, K., Toyama, K., and Shay, J. W. (1996). A nonradioactive, fluorescence-based telomeric repeat amplification protocol to detect and quantitate telomerase activity. *Trends Genet.* **12**: 395–396.
44. Smilenov, L. B., Morgan, S. E., Mellado, W., Sawant, S. G., Kastan, M. B., and Pandita, T. K. (1997). Molecular cloning and chromosomal localization of Chinese hamster telomeric protein chTRF1: Its potential role in chromosomal instability. *Oncogene* **17**: 2137–2142.
45. Powis, G., Bonjouklian, R., Berggren, M. M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W. F., and Dodge, J. (1994). Wortmannin, a potent and selective inhibitor of phosphatidylinositol 3-kinase. *Cancer Res.* **54**: 2419–2423.
46. Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirola, L., Vanhaesebroeck, B., Waterfield, M. D., and Panayotou, G. (1996). Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.* **16**: 1722–1733.
47. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269**: 5241–5248.
48. Brunn, G. J., Williams, J., Sabers, C., Wiederrecht, G., Lawrence, J. C., and Abraham, R. T. (1996). Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. *EMBO J.* **15**: 5256–5267.
49. Jiang, K., Coppola, D., Crespo, N., Nicosia, S. V., Hamilton, A., Sebt, S., and Cheng, J. Q. (2000). The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. *Mol. Cell. Biol.* **20**: 139–148.
50. Yuan, Z. Q., Sun, M., Feldman, R., Wang, G., Ma, X., Jiang, C., Coppola, D., Nicosia, S. V., and Cheng, J. Q. (2000). Frequent

- activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/AKT pathway in human ovarian cancer. *Oncogene* **19**: 2324–2330.
51. Ulaner, G. A., Hu, J. F., Vu, T. H., Giudice, L. C., and Hoffman, A. R. (1998). Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. *Cancer Res.* **58**: 4168–4172.
 52. Ambion (1998). The truth: Your RNA is contaminated How do you know if your RNA is contaminated with DNA! *TechNotes* **7**: 1–3.
 53. Ueda, M., Ouhit, A., Bito, T., Nakazawa, K., Lubbe, J., Ichihashi, M., Yamasaki, H., and Nakazawa, H. (1997). Evidence for UV-associated activation of telomerase in human skin. *Cancer Res.* **57**: 370–374.
 54. Hande, M. P., Balajee, A. S., and Natarajan, A. T. (1997). Induction of telomerase activity by UV-irradiation in Chinese hamster cells. *Oncogene* **15**: 1747–1742.
 55. Ong, A., Maines-Bandiera, S. L., Roskelley, C. D., and Auersperg, N. (2000). An ovarian adenocarcinoma line derived from SV40/E-cadherin-transfected normal human ovarian surface epithelium. *Int. J. Cancer* **85**: 430–437.
 56. Auersperg, N., Pan, J., Grove, B. D., Peterson, T., Fisher, J., Maines-Bandiera, S., Somasiri, A., and Roskelley, C. D. (1999). E-cadherin induces mesenchymal-to-epithelial transition in human ovarian surface epithelium. *Proc. Natl. Acad. Sci. USA* **96**: 6249–6254.
 57. Wong, A. S., Maines-Bandiera, S. L., Rosen, B., Wheelock, M. J., Johnson, K. R., Leung, P. C., Roskelley, C. D., and Auersperg, N. (1999). Constitutive and conditional cadherin expression in cultured human ovarian surface epithelium: Influence of family history of ovarian cancer. *Int. J. Cancer* **81**: 180–188.
 58. Ito, I., Miegishi, T., Fukuda, J., Shinozaki, H., and Auersperg, N. (2000). Presence of activin signal transduction in normal ovarian cells and epithelial ovarian carcinoma. *Brit. J. Cancer* **82**: 1415–1420.
 59. Cruet, S., Salamanca, C., Mitchell, G. W., and Auersperg, N. (1999). $\alpha V\beta 3$ and vitronectin expression by normal ovarian surface epithelial cells: Role in cell adhesion and cell proliferation. *Gynecol. Oncol.* **75**: 254–260.
 60. Maines-Bandiera, S. L., and Auersperg, N. (1997). Increased E-cadherin expression in ovarian surface epithelium: An early step in metaplasia and dysplasia? *Int. J. Gynecol. Path.*, **16**: 250–255, 1997.
 61. Sharma, S., Raymond, E., Soda, H., Izbic, E., Davidson, K., Lawrence, R., and Von Hoff, D. D. (1998). Dimethyl sulfoxide (DMSO) causes a reversible inhibition of telomerase activity in a Burkitt lymphoma cell line. *Leukemia Res.* **22**: 663–670.
 62. Vaziri, H., and Benchimol, S. (1998). Reconstitution of telomerase activity in normal cell leads to elongation of telomeres and extends replicative life span. *Curr. Biol.* **8**: 279–282.
 63. Morin, G. B. (1989). The human telomere terminal transferase is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell* **59**: 521–529.
 64. Shaw, M., Cohen, P., and Alessi, D. R. (1998). The activation of protein kinase B by H_2O_2 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2. *Biochem. J.* **336**: 241–246.
 65. Joo, O. H., Hande, M. P., Lansdorp, P. M., and Natarajan, A. T. (1998). Induction of telomerase activity and chromosome aberrations in human tumor cell lines following X-irradiation. *Mutat. Res.* **401**: 121–131.
 66. Yu, G. L., and Blackburn, E. H. (1991). Developmentally programmed healing of chromosomes by telomerase in tetrahymena. *Cell* **67**: 823–832.
 67. Ishibashi, T., and Lippard, S. J. (1998). Telomere loss in cells treated with cisplatin. *Proc. Natl. Acad. Sci. USA* **95**: 4219–4223.
 68. May, A., Nairn, R. S., Okumoto, D. S., Wassermann, K., Stevnsner, T., Jones, J. C., and Bohr, V. A. (1993). Repair of individual DNA strands in the hamster dihydrofolate reductase gene after treatment with ultraviolet light, alkylating agents, and cisplatin. *J. Biol. Chem.* **268**: 1650–1657.
 69. Faraoni, I., Turriziani, M., Masci, G., De Vecchis, L., Shay, J. W., Bonmassar, E., and Graziani, G. (1997). Decline in telomerase activity as a measure of tumor cell killing by antineoplastic agents in vitro. *Clin. Cancer Res.* **3**: 579–585.
 70. Andley, U. P., and Weber, J. G. (1995). Ultraviolet action spectra for photobiological effects in cultured human lens epithelial cells. *Photochem. Photobiol.* **62**: 840–846.
 71. Roshchupkin, D. I., Pelenitsyn, A. B., Potapenko, A. Y., Talitsky, V. V., and Valdimirov, Y. A. (1975). Study of the effects of ultraviolet light on biomembranes-IV. The effects of oxygen on UV-induced hemolysis and lipid photoperoxidation in rat erythrocytes and liposomes. *Photochem. Photobiol.* **21**: 63–69.
 72. Tapiero, H., Zwingelstein, G., Fourcade, A., and Prtoulkian, J. (1983). The effect of dimethyl sulfoxide on the membrane dynamics and the phospholipid composition of two different cell lines. *Ann. N. Y. Acad. Sci.* **411**: 383–388.
 73. de Bruijne, A. W., and van Stevenink, J. (1974). The influence of dimethylsulfoxide on the red cell membrane. *Biochem. Pharmacol.* **23**: 3247–3258.
 74. Yu, Z. W., and Quinn, P. J. (1998). The modulation of membrane structure and stability by demethylsulphoxide. *Mol. Membr. Biol.* **15**: 59–68.

Received July 3, 2000

Revised version received March 20, 2001

Published online May 24, 2001

Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer

Zeng Qiang Yuan¹, Mei Sun¹, Richard I Feldman², Gen Wang¹, Xiao-ling Ma¹, Chen Jiang¹, Domenico Coppola¹, Santo V Nicosia¹ and Jin Q Cheng^{*1}

¹Department of Pathology, H Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, Tampa, Florida, FL 33612, USA; ²Cancer Research Department, Berlex Biosciences, Richmond, California, CA 94804, USA

We previously demonstrated that AKT2, a member of protein kinase B family, is activated by a number of growth factors via Ras and PI 3-kinase signaling pathways. Here, we report the frequent activation of AKT2 in human primary ovarian cancer and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase (PI 3-kinase)/Akt pathway. *In vitro* AKT2 kinase assay analyses in 91 ovarian cancer specimens revealed elevated levels of AKT2 activity (>3-fold) in 33 cases (36.3%). The majority of tumors displaying activated AKT2 were high grade and stages III and IV. Immunostaining and Western blot analyses using a phospho-ser-473 Akt antibody that detects the activated form of AKT2 (AKT2 phosphorylated at serine-474) confirmed the frequent activation of AKT2 in ovarian cancer specimens. Phosphorylated AKT2 in tumor specimens localized to the cell membrane and cytoplasm but not the nucleus. To address the mechanism of AKT2 activation, we measured *in vitro* PI 3-kinase activity in 43 ovarian cancer specimens, including the 33 cases displaying elevated AKT2 activation. High levels of PI 3-kinase activity were observed in 20 cases, 15 of which also exhibited AKT2 activation. The remaining five cases displayed elevated AKT1 activation. Among the cases with elevated AKT2, but not PI 3-kinase activity (18 cases), three showed down-regulation of PTEN protein expression. Inhibition of PI 3-kinase/AKT2 by wortmannin or LY294002 induces apoptosis in ovarian cancer cells exhibiting activation of the PI 3-kinase/AKT2 pathway. These findings demonstrate for the first time that activation of AKT2 is a common occurrence in human ovarian cancer and that PI 3-kinase/Akt pathway may be an important target for ovarian cancer intervention. *Oncogene* (2000) 19, 2324–2330.

Keywords: Akt; PI 3-kinase; signal transduction; ovarian cancer

Akt/PKB represents a subfamily of the serine/threonine protein kinases (Bellacosa *et al.*, 1991; Jones *et al.*, 1991a, b; Cheng *et al.*, 1992; Konishi *et al.*, 1995; Nakatani *et al.*, 1999). Three members of this family, AKT1/PKB α , AKT2/PKB β and AKT3/PKB γ have been identified. Akt is activated by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress (Franke *et al.*,

1995; Burgering *et al.*, 1995; Shaw *et al.*, 1998; Liu *et al.*, 1998). Activation of Akt and AKT2 by growth factor is mediated by PI 3-kinase (Franke *et al.*, 1995; Meier *et al.*, 1997; Liu *et al.*, 1998). Active Ras and Src have also shown to activate Akt and AKT2 and this activation is blocked by wortmannin, a PI 3-kinase inhibitor, indicating that Ras and Src also mediate the activation of Akt and AKT2 and are located upstream of PI 3-kinase (Datta *et al.*, 1996; Liu *et al.*, 1998). Several lines of evidence suggest that PI 3-kinase regulates Akt activation through the following mechanism: the product of PI 3-kinase, phosphatidylinositol-3,4,5-trisphosphate, binds to the pleckstrin homology (PH) domain of Akt after growth factor stimulation, resulting in recruitment of Akt to the cell membrane. A conformational change of Akt follows, which allows residues Thr-308 and Ser-473 to be phosphorylated by upstream kinases, PDK-1 and PDK2 or ILK, respectively (Alessi and Cohen, 1998; Delcommenne *et al.*, 1998). Several downstream targets of Akt, each of which contains the Akt phosphorylation consensus sequence R-X-R-X-X-S/T-F/L, have been identified (Alessi and Cohen, 1998), pointing to the possible mechanisms by which Akt promotes cell survival and blocks apoptosis. One such target is GSK3. Akt phosphorylates GSK3 and leads to inactivation of GSK3, accumulation of β -catenin, and activation of *Myc* transcription. There is also evidences that Akt phosphorylates the proapoptotic proteins BAD, caspase-9 and transcription factor FKHR1, resulting in reduced binding of BAD to Bcl-X_L and inhibition of caspase-9 protease activity and Fas ligand transcription (Datta *et al.*, 1997; del Peso *et al.*, 1997; Cardone *et al.*, 1998; Brunet *et al.*, 1999). Recent studies demonstrated that PTEN/MMAC1 tumor suppressor and SHIP, tyrosine and inositol phosphatases, dephosphorylate phosphatidylinositol-3,4,5-triphosphate, thus, inhibiting the PI 3-kinase/Akt signaling pathway (Stambolic *et al.*, 1998; Aman *et al.*, 1998).

Several members within the PI 3-kinase/Akt pathway, including PI 3-kinase, PTEN, AKT2, and β -catenin, have been implicated in human neoplasms (Alessi and Cohen, 1998). Overexpression of p85, the regulatory subunit, or p110, the catalytic subunit, of PI 3-kinase is able to transform cells (Chang *et al.*, 1997; Jimenez *et al.*, 1998). Alterations of PI 3-kinase have also been detected in a number of human malignancies (Shayesteh *et al.*, 1999; Phillips *et al.*, 1998). Mutation and/or down regulation of the *Pten* are frequently observed in endometrial carcinoma, glioblastoma, breast cancer and prostate carcinoma (Stambolic *et*

*Correspondence: J Q Cheng

Received 10 January 2000; revised 23 March 2000; accepted 27 March 2000

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100

al., 1998). We and others have previously demonstrated alterations of *AKT2* at DNA, mRNA and/or protein level in several types of human malignancy (Cheng *et al.*, 1992, 1996; Bellacosa *et al.*, 1995; Ruggeri *et al.*, 1998). In particular, amplification/overexpression of *AKT2* has been detected in 10–20% of ovarian carcinomas and pancreatic cancers. However, increased activation of *AKT2* in human tumors has not been demonstrated previously.

In this report, we show that kinase activity of *AKT2* is frequently elevated in human primary ovarian tumor. *In vitro* *AKT2* kinase assays were performed in 91 primary human ovarian tumors, including 34 serous cystadenocarcinomas, four mucinous cystadenocarcinomas, 25 papillary serous adenocarcinomas, 10 endometrioid adenocarcinomas, two borderline tumors, five granulosa cell tumors and 11 other rare types of tumors including adenosarcoma, thecoma, fibroma, and mesodermal mixed tumor. Lysates from tumor specimens containing equivalent amounts of protein were precleared and incubated with anti-*AKT2* antibody, which specifically reacts with *AKT2*, in the presence of protein-A:protein-G beads. The immunoprecipitates were subjected to *in vitro* kinase assay using Histone H2B as the substrate. *In vitro* kinase assays were carried out three times for each specimen. Average reading of the kinase activity threefold higher than that in normal ovarian tissue was considered elevated *AKT2* activity. The results revealed an elevated level of *AKT2* kinase in 33 specimens (36.3%), including 15 serous cystadenocarcinomas (44%), 14 papillary serous adenocarcinomas (56%) and four other types of tumor including one adenosarcoma, two malignant clear cell tumors, and one mixed mullerian tumor. Examples of *AKT2* kinase activity in ovarian cancer are shown in Figure 1. Interestingly, the majority of cases exhibiting activation of *AKT2* were serous adenocarcinomas (29/33). None of endometrioid, borderline, mucinous, and granulosa cell tumors exhibited elevated *AKT2* kinase activity.

To examine whether variations in *AKT2* protein expression level contributes to the level of *AKT2* activity observed in ovarian tumor specimens, we

analysed its expression by Western blot analyses using a specific anti-*AKT2* antibody. A high level of *AKT2* protein was detected in 42 of 91 cases (46%). The 33 cases that exhibited elevated *AKT2* activity displayed variable levels of *AKT2* protein, 25 of which expressed *AKT2* at high or moderate levels (Figure 1 and Table 1). These results were also confirmed by immunohistochemical staining of the tumor tissue section (data not shown).

Previous studies demonstrated that phosphorylation of Thr-308 and Ser-473 of Akt1 is required for its full activation. Upon stimulation of PI 3-kinase by growth factors, Akt1 becomes phosphorylated at these two residues. It has been shown that *AKT2* is activated by phosphorylation of the equivalent residues (Thr-309 and Ser-474) (Alessi and Cohen, 1998). The homology of Akt1 to *AKT2* and *AKT3* is 90.4% and 87.8% at the amino acid level, respectively (Cheng *et al.*, 1992; Nakatani *et al.*, 1999), and the sequence of the serine-473 phosphorylation site of Akt1 is highly conserved in *AKT2* and *AKT3* (Figure 2a).

Anti-phospho-Ser473 Akt antibody has widely been used to identify Akt1 activation. To determine whether such an antibody can be used to detect *AKT2* activation in ovarian cancer specimens by Western blotting and immunostaining, we tested whether a phospho-Ser473 Akt antibody reacted with phosphorylated *AKT2*. HA-*AKT2* expression construct was transfected to COS-7 cells. After serum-starvation overnight and IGF-1 stimulation for 10 min, HA-*AKT2* was immunoprecipitated with anti-HA monoclonal antibody. The immunoprecipitates were separated by SDS-PAGE and probed with phospho-Ser473 Akt antibody. The results showed that the antibody against phospho-Ser473 Akt strongly reacted with phospho-Ser 474 of *AKT2* (Figure 2b).

We next examined *AKT2* phosphorylation by Western blot analyses of *AKT2* immunoprecipitates with phospho-Ser473 Akt antibody in 43 ovarian tumor specimens including the 33 cases with and 10 cases without elevated *AKT2* kinase activity. A sheep anti-*AKT2* antibody was used to incubate with equal amount of protein from ovarian tumor lysates in the presence of protein-A and protein-G agarose. After extensive wash with the lysis buffer, the *AKT2* immunoprecipitates were separated by SDS-PAGE and the blots were probed with the rabbit polyclonal phospho-Ser473 Akt antibody (New England Biolab). Phosphorylated *AKT2* was detected only in ovarian tumors with elevated *AKT2* kinase activity; for example, see data from tumors T2, 5, 7, 10 and 13, which exhibit high *AKT2* kinase activity, shown in Figure 2c. These data confirm the results obtained from *in vitro* *AKT2* kinase assay and indicate that *AKT2* activity is regulated by phosphorylation at Ser474 in human primary tumors.

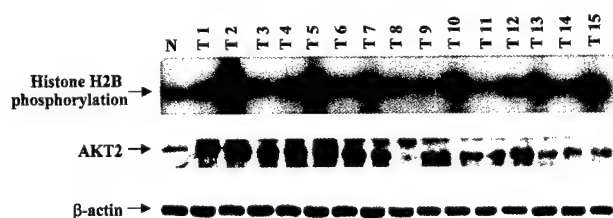


Figure 1 Elevated level of *AKT2* kinase activity in ovarian cancer. Top panel: *In vitro* kinase assays of *AKT2* immunoprecipitated from 15 frozen representative tumor specimens. Frozen tissues from ovarian carcinoma was mechanically smashed in liquid nitrogen and lysed by a Tissue Tearor in a lysis buffer (Liu *et al.*, 1998). Lysates were incubated with anti-*AKT2* antibody in the presence of protein A-protein G (2:1) agarose beads for 2 h at 4°C. Following extensive wash, immunoprecipitates were subjected to *in vitro* kinase assay (Liu *et al.*, 1998). Histone H2B was used as the exogenous substrate. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Phosphorimager. *AKT2* is activated in cases T2, T5, T7, T10, T13, and T15. Middle panel: Western blot analysis of *AKT2* expression in ovarian carcinoma. The blot was reprobed with anti- β -actin antibody (Bottom panel)

Table 1 *AKT2* activation and clinical stage

Stage	n	<i>AKT2</i> kinase activity		<i>AKT2</i> protein level	
		Normal	High	Low	High/moderate
I	7	7	0	5	2
II	11	11	0	6	5
IIIA	5	5	0	1	4
IIIC	53	27	26	31	22
IV	15	8	7	6	9

Due to the fact that stromal tissues approximately account for 20~30% of the tumor specimens used in this study, we examined whether activation of AKT2 is derived from tumor cells or stromal tissues by immunostaining the tumor paraffin sections with phospho-Ser473 Akt antibody. We first tested if the phospho-Ser473 Akt antibody is capable of recognizing phosphorylated AKT2 by immunohistochemistry. Cell paraffin blocks were prepared from serum-starved and serum-starved/EGF-stimulated OVCAR-3 cells that overexpress AKT2 (Cheng *et al.*, 1992). Phospho-Ser473 antibody was used to detect phosphorylated AKT2 in the section from these blocks by immunohistochemical means. Strong positive staining was observed in EGF-stimulated but not serum-starved OVCAR-3 cells (panel 1~2 in Figure 3a). Phosphorylation status of AKT2 in these cells was confirmed by Western blot analysis with phospho-S473 Akt antibody (lanes 1 and 2 in Figure 3b).

Immunostaining of the tumor paraffin sections with phospho-Ser473 antibody was performed in 43 ovarian cancer specimens including 33 cases exhibiting elevated AKT2 kinase activity and 10 cases without AKT2 activation. Positive staining of tumor cells was

observed in 38 cases including 33 with and five without AKT2 activation detected by *in vitro* kinase assay and Western blot analyses. The fact that phospho-Ser473 Akt antibody recognizes Akt1, AKT2 and possible AKT3 suggests that activation of AKT1 or AKT3 present in these five cases, which may be due to different expression levels of three isoforms of Akt. Immunohistochemical staining of paraffin tissue sections with anti-AKT1 and -AKT2 antibodies revealed that AKT1 but not AKT2 is highly expressed in these five specimens (Figure 3c). Moreover, *in vitro* kinase assay showed that AKT1 kinase activity is elevated in these specimens (Figure 3d, and also see case 8 in Figures 1 and 4). Unfortunately, no good AKT3 antibody is commercially available at present time, therefore, we could not determine if AKT3 protein is altered in these specimens. Strikingly, phosphorylated Akt localizes to cytoplasm and cell membrane. Even in EGF-stimulated OVCAR-3 cells, activated Akt stays in cytoplasm and cell membrane. No nuclear staining was observed (panels 2 and 4 of Figure 3a).

One mechanism that could result in an increased activation of AKT2 is an up-regulation of PI 3-kinase. PI 3-kinase is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit. Three isoforms of p85 and p110 have been cloned, namely p85 α , p85 β , p85 γ , p110 α , p110 β and p110 γ . Recent studies demonstrated transforming activity of p110 and p85 and frequent activation PI 3-kinase in colon and ovarian carcinoma cell lines (Chang *et al.*, 1997; Jimenez *et al.*, 1998; Shayesteh *et al.*, 1999; Phillips *et al.*, 1998). To examine PI 3-kinase activity in tumor tissues, immunoprecipitation with a pan-p85 specific antibody was performed in 43 ovarian cancer cases, including 33 with and 10 without AKT2 activation (Klippel *et al.*, 1993). Following extensive wash, the immunoprecipitates were subjected to *in vitro* PI 3-kinase assay using L- α -phosphatidylinositol-4,5-bis phosphate (PI-4,5-P₂) or L- α -phosphatidylinositol-4-phosphate (PI-4-P₁) as substrates. The conversion of PI-4,5-P₂ to PI-3,4,5-P₃ or PI-4-P₁ to PI-3,4-P₂ was determined by autoradiography and quantitated by Phosphorimager. Elevated PI 3-kinase activity was detected in 20 ovarian cancer specimens, 15 of which exhibited AKT2 activation and the other five displayed AKT1 activation (Figures 3 and 4). Since p110 α has been shown to be over-expressed and activated in ovarian cancer cell lines (Shayesteh *et al.*, 1999), we examined whether increased levels of activity observed in our primary ovarian tumor specimens resulted from increased levels of p110 expression. PI 3-kinase was immunoprecipitated with an anti-p85 antibody from equal amounts of tumor extract protein. The immunoprecipitates were then separated on a SDS-PAGE, transferred to a membrane and probed with a specific anti-p110 α antibody. Figure 4 shows a correlation between p110 α expression levels and PI 3-kinase activity in tumor samples. Quantitative analysis by a BioImager (Genomic Solutions Inc.) revealed that the levels of p110 α expression in the tumors with PI 3-kinase activation are 2~4-folds higher than that in the specimens showing no elevated PI 3-kinase, except T12 that exhibited high level of p110 α protein but no significant PI 3-kinase activation. These data indicate that increases of PI 3-kinase activity contribute to AKT activation in human primary ovarian carcinoma.

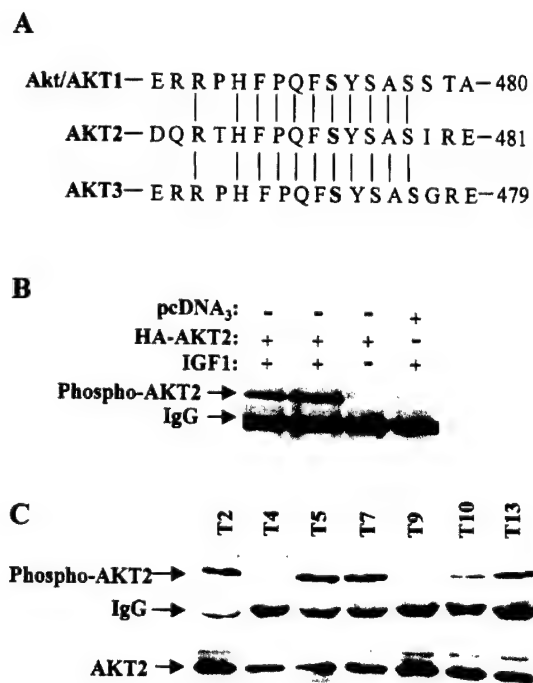


Figure 2 Phospho-Akt-Ser473 antibody recognizes phosphorylation form of AKT2 that is detected in ovarian cancer. (a) Comparison of C-terminal amino acid sequence of AKT1 and AKT2 and AKT3. Phosphorylation of serine-473 (AKT1) is well conserved in AKT2 (Ser-474) and AKT3 (Ser-472). (b) Western blot analysis of HA-AKT2 immunoprecipitates from COS-7 cells transfected with pcDNA₃-HA-AKT2 or pcDNA₃ vector alone. After transfection, the cells were serum-starved overnight and stimulated with IGF-1 for 10 min (lanes 1 and 2 from left) prior to harvesting cells. Immunoprecipitation was carried out with anti-HA monoclonal antibody and separated by SDS-PAGE. The blot was detected with Phospho-Akt-Ser473 antibody. (c) Western blot analysis of phosphorylation of AKT2 in ovarian cancer specimens. The tumor lysates were incubated with anti-AKT2 antibody. The resulting immunoprecipitates were separated by SDS-PAGE and detected with polyclonal phospho-Ser473 Akt antibody (upper panel) or polyclonal anti-AKT2 antibody (lower panel).

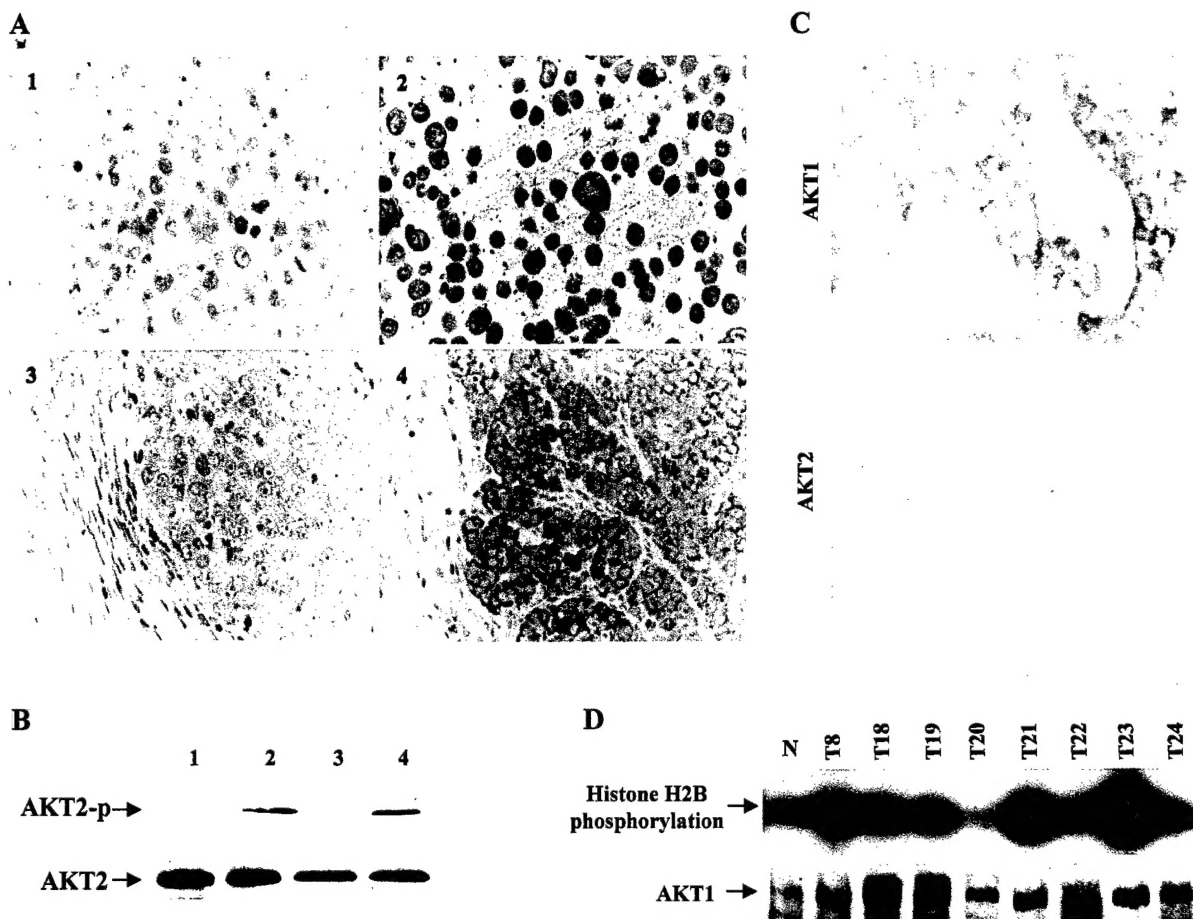


Figure 3 Phosphorylated Akt detected by immunostaining localizes to cell membrane and cytoplasm of ovarian tumor cells. The paraffin sections were subjected to antigen retrieval by boiling in a microwave and then incubated in a blocking solution and an avidin/biotin blocking kit (Vector). The primary antibody to phospho-S473 Akt (Upstate Biotechnology) was applied at a dilution of 1:200. After incubation, the slides were treated with biotinylated rabbit anti-goat immunoglobulin and streptavidin and biotinylated alkaline phosphatase. (a) Sections of paraffin blocks prepared from serum-starved (1), EGF-stimulated OVCAR3 cells (2) and 2 primary ovarian carcinoma specimens (3~4) were stained with phospho-Ser473 antibody. No staining was observed in serum starved OVCAR3 cells and a specimen without elevated AKT2 kinase activity (1 and 3), whereas strong reaction with phospho-Ser473 antibody is seen in EGF-stimulated OVCAR3 cells and a tumor specimen exhibiting AKT2 activation. (b) Western blot analysis of the AKT2 immunoprecipitates from the cells and the tissues that are used for immunostaining in Figure 3a. The filter was detected with phospho-Ser473 antibody (upper panel) and anti-AKT2 antibody (lower panel). (c) Immunohistochemical staining of an ovarian cancer specimen with anti-AKT1, and -AKT2 antibodies. (d) *In vitro* kinase assay (upper panel) and Western blot (bottom panel) of AKT1 immunoprecipitates from frozen tumor specimens. Results represent three independent experiments. AKT1 is activated in cases T8, T18, T19, T21, and T23

Another mechanism shown to result in activation of Akt is inactivation or loss of expression of PTEN. Inactivating mutations of the *Pten* tumor suppressor gene, on chromosome 10q23, have been described in prostate, endometrial and ovarian endometrioid carcinomas (Obata *et al.*, 1998). Previous studies have also observed down-regulation of PTEN protein in prostate cancer (Wu *et al.*, 1998; McMenamin *et al.*, 1999). Therefore, we examined whether down-regulation of PTEN is associated with AKT2 activation in ovarian cancer. We analysed PTEN expression in 18 ovarian cancer specimens displaying elevated AKT2 activity by Western blot. A large reduction in PTEN protein expression was observed in three cases, two of which were serous cystadenocarcinomas and one was a papillary serous adenocarcinoma (Figure 5). In these cases, we did not detect either mutations or low mRNA level of the *Pten* gene (data not shown). Interestingly, we failed to observe elevated AKT2 kinase activity in 10 endometrioid adenocarcinomas examined, even though PTEN mutations have most

frequently been detected in this type of ovarian tumor. These results suggest that down-regulation of PTEN protein expression, either by translational or post-translational changes, may be a factor in elevating AKT2 activation in non-endometrioid ovarian cancer.

We next examined the relationships between activation of AKT2 and tumor stage and grade. The results are presented in Tables 1 and 2. High levels of AKT2 activity were seen in stages III and IV (33/68, 48.5%) but not stages I and II. The incidence of AKT2 activation increased with increasing grade. High levels of AKT2 kinase activity were detected in 52.9% of grades III and IV tumors, whereas activation of AKT2 was observed in only 15% of grades I and II tumors. These data indicate that activation of AKT2 in ovarian carcinoma is associated with late stage and high-grade tumors, and suggest an increase in the activation of AKT2 kinase as ovarian cancer progresses to a more aggressive phenotype.

It has been shown that Akt induces cell survival and suppresses the apoptotic death of a number of cell types induced by a variety of stimuli, including growth factor



Figure 4 Activation of PI 3-kinase in human ovarian cancer specimens. *In vitro* PI 3-kinase assay of the anti-p85 immunoprecipitates from 12 frozen ovarian cancer specimens. The tumor tissue lysates were immunoprecipitated with pan-p85 antibody (Santa Cruz). Following wash, the presence of PI 3-kinase activity in immunoprecipitates was determined by incubating the beads with reaction buffer. Phospholipids were extracted and separated by thin-layer chromatography as previously described (Jiang *et al.*, 2000). The conversion of PI-4,5-P₂ to PI 3-phosphate and PI-4-P to PI-3,4-P₂ was determined by autoradiography and quantitated by using a Phosphorimager. Elevated levels of PI 3-kinase activity (top panel) and p110 α protein (middle panel) are detected in cases 2, 7, 8, 12, and 13. The expression levels of p110 α are quantified by a BioImager. The density of each band is indicated at the bottom

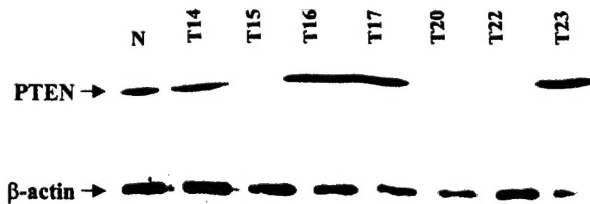


Figure 5 Down regulation of PTEN in human ovarian carcinoma. Western blot analyses of the tumor tissue lysates with anti PTEN (upper) and β -actin (bottom) antibody. No detectable level of PTEN was observed in three cases

Table 2 AKT2 activation and grade

Grade	n	AKT2 kinase activity	
		Normal	High
1	21	17	4
2	19	17	2
3	49	24	25
4	2	0	2

withdrawal, cell-cycle discordance and loss of cell adhesion. To assess the influence of PI 3-kinase/AKT activation on the cell growth of human ovarian cancer cells, we have performed *in vitro* kinase assay and observed activation of AKT2 and PI 3-kinase in 2 of 5 ovarian cancer cell lines (Figure 6a). If PI 3-kinase/Akt pathway is crucial for survival in such ovarian tumor cells, blocking the activity of PI 3-kinase/Akt is expected to inhibit the cell growth and/or induce apoptosis. To test this hypothesis, three ovarian cancer cell lines with or without PI 3-kinase/AKT2 activation were treated with PI 3-kinase inhibitors, wortmannin (200 nM) or LY294002 (40 μ M), or vehicle (DMSO), for 12 h in a medium containing 1% fetal calf serum. Those cell lines exhibiting elevated levels of PI 3-kinase and AKT2 activity underwent apoptosis after treatment with wortmannin or LY294002, whereas no apoptosis was detected in the cell line (e.g., A2780) without PI 3-kinase/AKT2 activation (Figure 6).

In this study, we have demonstrated frequent activation of AKT2 kinase in human primary ovarian cancer by *in vitro* kinase assay, Western blot and immunohistochemical staining. Phosphorylated AKT2 has a cell membrane and cytoplasmic but not nuclear localization. Increased PI 3-kinase activation is observed in the majority of cases displaying AKT2 activation. High levels of AKT2 protein expression are also frequently detected in ovarian cancer specimens. Down regulation of PTEN was observed in non-endometrioid ovarian tumors, which may contribute to AKT2 activation. Moreover, activation of AKT2 is associated with high grade and late stage ovarian cancer and direct inhibition of PI 3-kinase/Akt pathway induces apoptosis in ovarian cancer cell lines exhibiting activation of PI 3-kinase and AKT2.

Previous studies showed that alteration of oncogene could occur at DNA, mRNA, protein, or enzymatic level. An extensive review of the topic indicates that the frequency of oncogene amplification in primary tumors is about 5–38%, depending on tumor type, stage, and grade as well as the individual gene (Brison, 1993). The incidence of overexpression of some oncogenes in certain tumors is even higher due to the mechanism of enhanced transcription or translation, and mRNA or protein stabilization (Brison, 1993; Devilee and Cornelisse, 1994; Berns *et al.*, 1995). We and others have previously shown alterations of AKT2 at DNA and mRNA levels in ~12% and ~25% of ovarian carcinoma, respectively (Cheng *et al.*, 1992; Bellacosa *et al.*, 1995). In this study, we demonstrate activation of AKT2 kinase in 36.3% of primary ovarian tumors with highest frequency (49%) in serous adenocarcinomas. Moreover, we have also observed overexpression of AKT2 protein in 46% of ovarian cancer specimens examined. These data, as compared to AKT2 alteration at DNA and RNA levels, indicate that activation and overexpression of AKT2 protein by aberrant translational regulation and posttranslational modification of AKT2 is a more common occurrence in human ovarian cancer. The fact that overexpression of wild type AKT2 in NIH3T3 cells results in malignant transformation (Cheng *et al.*, 1997) suggests that activation and overexpression of AKT2 in human ovarian carcinoma could play an important role in the development of this malignancy.

Several studies demonstrated that ectopically expressed Akt1 and AKT2, following growth factor stimulation, initially locates to cell membrane and then translocates to the nucleus (Andjelkovic *et al.*, 1997; Meier *et al.*, 1997). This is thought to allow Akt mediated phosphorylation of nuclear transcription factors, such as Forkhead proteins and CREB (Brunet *et al.*, 1999; Kops *et al.*, 1999; Du and Montminy, 1998). In contrast, in primary ovarian tumor specimens examined, we found that phosphorylated AKT2 localizes to cell membrane and cytoplasm but not the nucleus. Moreover, activated endogenous AKT2 in AKT2-overexpressing OVCAR cells was also localized to cell membrane and cytoplasm only. These conflicting observations may be due to the fact that ectopic overexpression of Akt affects its subcellular localization.

We and others have previously documented that AKT2 is a downstream target of PI 3-kinase and is activated by a number of growth factors (Meier *et al.*,

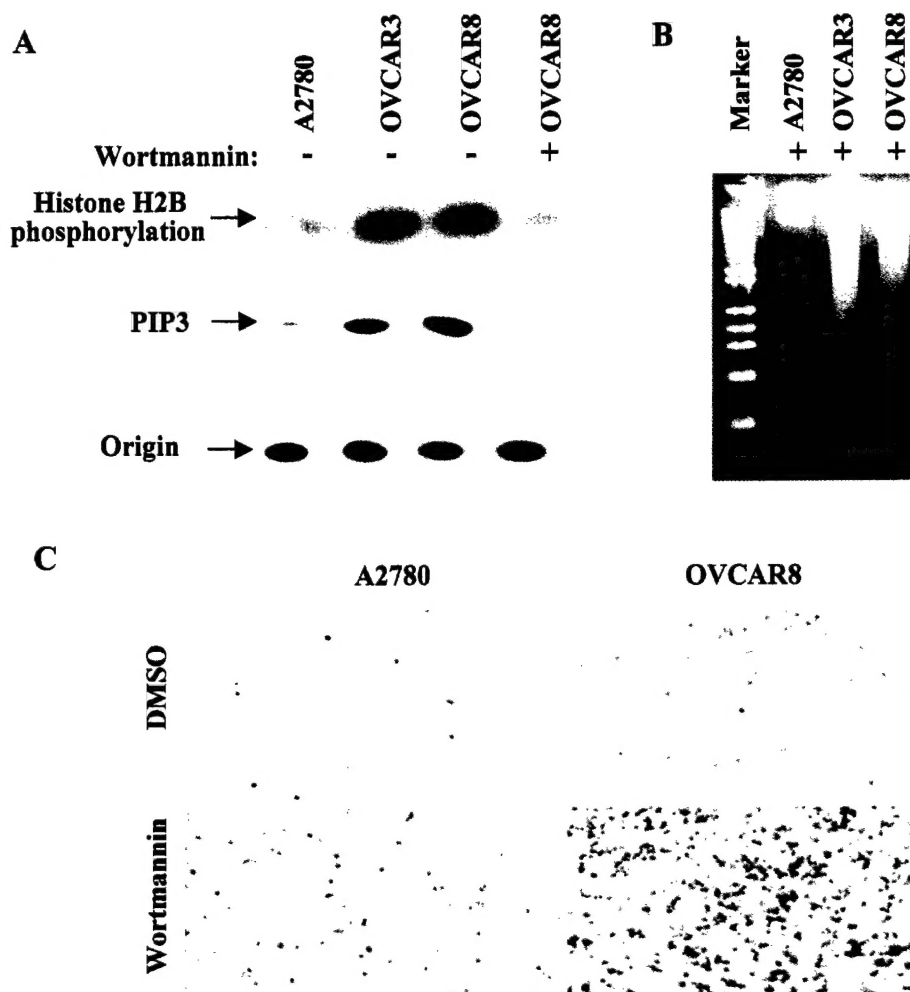


Figure 6 Inhibition of PI 3-kinase/AKT2 activity induces apoptosis in PI 3-kinase/AKT2-activating ovarian cancer cell lines. (a) *In vitro* Akt and PI 3-kinase assay of the immunoprecipitates from A2780, OVCAR3 and OVCAR8 cells. AKT2 (upper panel) and PI 3-kinase (bottom panel) are activated in OVCAR3 and OVCAR8 cells. (b) and (c) DNA fragmentation and TUNEL assay showing that Wortmannin blocks activity of PI 3-kinase and AKT2, and induces apoptosis in OVCAR3 and OVCAR8 cells. The cells were seeded into 60 mm dishes and grown for 24 h and then treated with wortmannin (200 nM) or LY294002 (40 μ M) for 12 h. Apoptosis was determined by TdT-mediated dUTP nick end labeling (TUNEL) using an *in situ* cell death detection kit (Boehringer). To detect DNA fragmentation, cellular DNA was prepared using the DNA kit (Qiagen). The DNA was analysed on 1.5% agarose gel and visualized by ethidium bromide staining

1997; Liu *et al.*, 1998). Active Ras and Src significantly induce AKT2 activation (Liu *et al.*, 1998). Recent studies have shown that Akt activity is regulated by PTEN, which reduces intracellular levels of PI-3,4,5-P₃ in cells by converting PI-3,4,5-P₃ to PI-4,5-P₂ and, thus, inhibits PI 3-kinase/Akt signaling pathway (Stambolic *et al.*, 1998; Aman *et al.*, 1998). Therefore, AKT2 activation in human ovarian cancer may result from; (a) PI 3-kinase activation; (b) *Pten* mutation; (c) overexpression of AKT2; (d) alterations of growth factor receptor, such as overexpression or mutation of EGFR; (e) *ras* mutation; or (f) active mutation of the *AKT2* gene. In this report, we document that nearly half of cases with AKT2 activation (15/33) display PI 3-kinase activation, which supports a recent observation of PI 3-kinase activation in ovarian cancer cell lines (Shayesteh *et al.*, 1999). Down regulation of PTEN was detected in three non-endometrioid cases of ovarian tumors. Interestingly, we did not observe either lack of PTEN expression or AKT2 activation in all 10 endometrioid adenocarcinomas examined, implying that the *Pten* may not be mutated in these specimens. We have also performed single strain conformation

polymorphism analyses of the tumors carrying activated AKT2. No *AKT2* mutation was observed. The cases exhibiting AKT2 activation express variable levels of AKT2 protein, suggesting that expression of AKT2 protein is required for activation of AKT2 in ovarian tumor.

The fact that three members of Akt family are downstream targets of PI 3-kinase and are regulated by similar mechanism suggests that AKT1 and AKT3 may also be activated in human ovarian cancer. In this study, we focused on activation of AKT2 in primary ovarian cancer. However, we have shown activation of AKT1 and PI 3-kinase but not AKT2 in five ovarian cancer specimens, which express high levels of AKT1 but very low levels of AKT2 (Figure 3). Additional experiments to evaluate the significance of this finding are in progress.

We have previously demonstrated that an activated Ras significantly activates AKT2. We have also recently documented that farnesyltransferase inhibitor (FTI)-277, an anticancer drug blocking the posttranslational farnesylation of oncogenic Ras, inhibits PI 3-kinase and AKT2 activity and induces apoptosis in

AKT2 overexpressing cell lines (Jiang *et al.*, 2000). Overexpression of AKT2, but not Ras, sensitizes NIH3T3 cells to FTI-277. However, FTI-277 is not a direct inhibitor of PI 3-kinase and AKT2. In this study, we demonstrate that direct inhibition of PI 3-kinase/Akt pathway by wortmannin or LY294002 induces apoptosis in ovarian cancer cell lines with PI 3-kinase/AKT2 activation. Moreover, we have also shown that the majority of tumors with activated AKT2 are high grade and late stage. These data indicate that activation of PI 3-kinase/Akt may play a pivotal role in development of human ovarian cancer, especially in tumor progression, and that PI 3-kinase/Akt pathway could be an important target for intervention of this malignancy. Future studies are

required to define the role of Akt activation in ovarian malignant transformation.

Acknowledgments

We are grateful to Dr Sue A Shelley for critical reading and comments on the manuscript; June E Paciga, and Jie-liu Tang for technical support. We are also grateful for Tissue Procurement, DNA Sequence and Tissue Pathology Core Facilities at H Lee Moffitt Cancer Center for providing cancer specimens, sequencing and immunohistochemical staining. This work was supported by National Cancer Institute Grant CA77935 (to JQ Cheng) and University of South Florida Ovarian Cancer Pathobiology Grant 6115-000-20 (to SV Nicosia).

References

- Alessi DR and Cohen P. (1998). *Curr. Opin. Genet. Dev.*, **8**, 55–62.
- Aman MJ, Lamkin TD, Okada H, Kurosaki T and Ravichandran KS. (1998). *J. Biol. Chem.*, **273**, 33922–33928.
- Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM and Hemmings BA. (1997). *J. Biol. Chem.*, **272**, 31515–31524.
- Bellacosa A, Testa JR, Staal SP and Tsichlis PN. (1991). *Science*, **254**, 274–277.
- Bellacosa A, Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare D, Wan M, Dubeau L, Scambia G, Masciullo V, Ferrandina G, Panici PB, Mancuso S, Neri G and Testa JR. (1995). *Int. J. Cancer*, **64**, 280–285.
- Berns EMJJ, Foekens JA, van Staveren IL, van Putten WLJ, de Koning HYWCM, Portengen H and Klijin JGM. (1995). *Gene*, **159**, 11–18.
- Brison O. (1993). *Biochim. Biophys. Acta*, **1155**, 25–41.
- Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J and Greenberg ME. (1999). *Cell*, **96**, 857–868.
- Burgering BMT and Coffer PJ. (1995). *Nature*, **376**, 599–602.
- Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S and Reed JC. (1998). *Science*, **282**, 1318–1321.
- Chang HW, Aoki M, Fruman D, Auger KR, Bellacosa A, Tsichlis PN, Cantley LC, Roberts TM and Vogt PK. (1997). *Science*, **276**, 1848–1850.
- Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN and Testa JR. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 9267–9271.
- Cheng JQ, Ruggeri B, Klein WM, Sonoda G, Altomare DA, Watson DK and Testa JR. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 3636–3641.
- Cheng JQ, Altomare DA, Klein MA, Lee W-C, Mysliwiec T, Lissy NA and Testa JR. (1997). *Oncogene*, **14**, 2793–2801.
- Datta K, Bellacosa A, Chan TO and Tsichlis PN. (1996). *J. Biol. Chem.*, **271**, 30835–30839.
- Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y and Greenberg ME. (1997). *Cell*, **91**, 231–241.
- Delcommenne M, Tan C, Gray V, Rue L, Woodgett J and Dedhar S. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 11211–11216.
- del Peso L, Gonzalez-Garcia M, Page C, Herrera CR and Nunez G. (1997). *Science*, **278**, 687–689.
- Devilee P and Cornelisse CJ. (1994). *Biochim. Biophys. Acta*, **1198**, 113–130.
- Du K and Montminy M. (1998). *J. Biol. Chem.*, **273**, 32377–32379.
- Franke TF, Yang SL, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR and Tsichlis PN. (1995). *Cell*, **81**, 727–736.
- Jiang K, Coppola D, Crespo NC, Nicosia SV, Hamilton AD, Sefti SM and Cheng JQ. (2000). *Mol. Cell. Biol.*, **20**, 139–148.
- Jimenez C, Jones DR, Rodriguez-Viciano P, Gonzalez-Garcia A, Leonardo E, Wennstrom S, von Kobbe C, Toran JL, R-Borlado L, Calvo V, Copin SG, Albar JP, Gaspar ML, Diez E, Marcos MA, Downward J, Martinez-A C, Merida I and Carrera AC. (1998). *EMBO J.*, **17**, 743–753.
- Jones PF, Jakubowicz T, Pitossi FJ, Maurer F and Hemmings BA. (1991a). *Proc. Natl. Acad. Sci. USA*, **88**, 4171–4175.
- Jones PF, Jakubowicz T and Hemmings BA. (1991b). *Cell Reg.*, **2**, 1001–1009.
- Klippel A, Escobedo JA, Hu Q and Williams LT. (1993). *Mol. Cell. Biol.*, **13**, 5560–5566.
- Konishi H, Kuroda S, Tanaka M, Ono Y, Kameyama K, Haga T and Kikkawa U. (1995). *Biochem. Biophys. Res. Commun.*, **216**, 526–534.
- Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL and Burgering BM. (1999). *Nature*, **398**, 630–634.
- Liu A-X, Testa JR, Hamilton TC, Jove R, Nicosia SV and Cheng JQ. (1998). *Cancer Res.*, **58**, 2973–2977.
- McMenamin ME, Soung P, Perera S, Kaplan I, Loda M and Sellers WR. (1999). *Cancer Res.*, **59**, 4291–4296.
- Meier R, Alessi DR, Cron P, Andjelkovic M and Hemmings BA. (1997). *J. Biol. Chem.*, **272**, 30491–30497.
- Nakatani K, Sakaue H, Thompson DA, Weigel RJ and Roth RA. (1999). *Biochem. Biophys. Res. Commun.*, **257**, 906–910.
- Obata K, Morland SJ, Watson RH, Hitchcock A, Chenevix-Trench G, Thomas EJ and Campbell IG. (1998). *Cancer Res.*, **58**, 2095–2097.
- Phillips WA, St. Clair F, Munday AD, Thomas RJ and Mitchell CA. (1998). *Cancer*, **83**, 41–47.
- Ruggeri B, Huang L, Wood M, Cheng JQ and Testa JR. (1998). *Mol. Carcinogenesis*, **21**, 81–86.
- Shaw M, Cohen P and Alessi DR. (1998). *Biochem. J.*, **336**, 241–246.
- Shayesteh L, Lu YL, Kuo WL, Baldocchi R, Godfrey T, Collins C, Pinkel D, Powell B, Mills GB and Gray JW. (1999). *Nature Genet.*, **21**, 99–102.
- Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP and Mak TW. (1998). *Cell*, **95**, 29–39.
- Wu X, Senechal K, Neshat MS, Whang YE and Sawyers CL. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 15587–15591.